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In this progress report, we describe work during the past year (7/15/96-7/15/97) in studying different aspects of the signaling pathways initiated by the EGF receptor and the related Neu/ErbB2 tyrosine kinase. We describe a novel mechanism for growth factor signaling where growth factor-stimulated dimerization events (e.g. between the EGF receptor and Neu or between Neu and ErbB3) are transient and give rise to secondary receptor dimers. We also have obtained increasing evidence for the importance of two new signaling molecules, the c-Cbl protein and an 18 kDa splicing factor in the actions of the EGF receptor and Neu.

In the coming year, we intend to extend these studies and identify and characterize signaling molecules that are activated in human breast cancer cells following primary and secondary receptor dimerization events. We also will determine whether the Cbl proto-oncogene product serves to interface the EGF receptor with the Cdc42 GTP-binding protein in breast cancer cells and how the splicing factor, CBP20, is activated by heregulin. We also will determine if identical signaling pathways are operating in dog mammary carcinomas as an important step toward validating this system as a model for human breast cancer.

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7/8/97
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INTRODUCTION

The primary goals of the original proposal were to understand in some detail how members of the EGF receptor family become activated, with a primary emphasis being the Neu/ErbB2/HER2 tyrosine kinase, and then to establish the signal transduction pathways that are initiated by these activated receptors. The hope was to obtain new information regarding signaling pathways, which when aberrantly regulated may give rise to the development of breast cancer. Within this section of the annual report (for the period of July 15, 1996 through July 15, 1997), I will discuss some appropriate background for the research problem and review the purpose of the proposed research (including the methods of approach that are being used).

1. Background of the Research Problem.

The epidermal growth factor receptor (EGFR) family includes the EGFR, the Neu/ErbB2/Her2 tyrosine kinase (from here on referred to as Neu), and the ErbB3 and ErbB4 proteins (1-7). These receptors (M_r ~180 kDa) are all comprised of a single membrane-spanning helix that connects an appreciable extracellular (growth factor-binding) domain to a cytoplasmic tyrosine kinase domain. There have been a variety of lines of evidence that have implicated the EGFR family in different types of cancer. For example, the amplification of the EGFR gene has been found in squamous cell carcinomas, bladder, lung, liver, and stomach cancers, and in glioblastomas (8,9). Over-expressed Neu has been detected in a significant proportion of human breast cancers (up to 30-40%) and has a significant predictive value for the severity of the prognosis for patients with this disease (10-18). The ErbB3 protein may also be associated with human malignancy since as much as 100 fold increases in the levels of the *erbB3* transcript have been observed in human mammary carcinomas (5) and, likewise, ErbB4 appears to be over-expressed in some mammary adenocarcinoma cells and in neuroblastomas (7).

It appears to be well accepted that the dimerization of EGFR family members is a critical step in the initiation of their signaling pathways. The importance of receptor dimerization appears to lie in intermolecular (trans) phosphorylation events which enable the binding of specific cellular phospho-substrates and the activation of signaling cascades to the nucleus. A particularly interesting feature of the EGFR family is that both homodimerization and heterodimerization events occur. Thus, much of this original proposal was built on the finding (19,20) that Neu tyrosine kinase activity is stimulated by the growth factor, heregulin (21) [also known as Neu-differentiation-factor (NDF) (22-24), glial growth factor (25), ARIA (26), and neuregulin (27,28)] as an outcome of heregulin-promoted heterodimer formation between ErbB3 or ErbB4 and Neu. Overall, there are a number of possible combinations for receptor homo- and heterodimers, including EGFR-EGFR, EGFR-Neu, EGFR-ErbB3, Neu-ErbB3, Neu-ErbB4, ErbB3-ErbB4, and EGFR-ErbB4, thereby significantly increasing the diversity of signaling activities that can be stimulated by a specific growth factor. Recently, we have found that the situation is even more complicated, because in many cases the formation of the receptor dimer is a transient, rather than a stable, process (see "Progress Report"). This leads to the possibility of receptor dimerization cascades, such that an EGF-stimulated heterodimer comprised of the EGFR and Neu can dissociate, following EGFR activation and its trans-phosphorylation of Neu, and the phosphorylated/activated Neu molecule can then interact with other members of the EGFR family to form secondary dimers. As will be covered in more detail below, this further adds to the diversity of signaling events that can be triggered by a single growth factor binding interaction.

Of course a critical question concerns the identity of the signaling cascades that are triggered by these different receptor dimerization events. During the past year, we have begun to gather some interesting new information regarding two types of pathways, one which is initiated by EGF and a second which is initiated (preferentially) by heregulin. The EGF-stimulated pathway appears to center on a cellular proto-oncogene product called Cbl (i.e. the proto-oncogenic form for the Casitas B-lineage Lymphoma NS-1 retrovirus) (29). At present, little is known regarding how Cbl is regulated; however, during the past year we have begun to obtain some clues regarding how Cbl might participate in EGFR signaling. The heregulin-stimulated signaling pathway results in the activation of an 18 kDa protein that we initially identified based on its ability

to be photo-labeled with [α^{32}P]GTP in a heregulin-dependent manner. We have recently determined that this protein is identical to CBP20, an 18 kDa protein that (together with an 80 kDa protein CBP80) is involved in precursor messenger RNA splicing by binding the methyl guanosine cap on RNA molecules (30). This now raises a number of exciting possibilities regarding a heregulin-stimulated signaling pathway that specifically influences the splicing reaction and the profile of mRNA transcripts that are generated.

2. Purpose of the Present Research/Experimental Methods- The research goals for the coming year will continue to focus on understanding the mechanisms of activation of Neu and other members of the EGFR family and delineating their signaling pathways that lead to the nucleus. The original research proposal submitted to the Department of Defense was based on our finding that heregulin stimulates Neu tyrosine kinase activity by first binding to the ErbB3 protein (19). This then promotes the formation of an ErbB2-ErbB3 heterodimer and results in the activation of Neu and the trans-phosphorylation of ErbB3. A similar mechanism was proposed for heregulin binding to ErbB4 and stimulating the formation of ErbB2-ErbB4 heterodimers (20); however, an interesting difference is that while ErbB4 is capable of tyrosine kinase activity, ErbB3 is kinase-defective (31). Thus, ErbB3 serves as an adaptor molecule, imparting heregulin sensitivity to Neu and receiving phosphorylation signals to recruit cellular phospho-substrates.

During the past year, we have continued to gather evidence suggesting that these receptor heterodimerization events are more complicated than originally expected. In particular, we have made the unanticipated finding that the formation of heterodimers between members of the EGFR family is transient, such that following tyrosine kinase activation and trans-phosphorylation, the receptor dimers dissociate into their component monomeric receptors. The dynamic nature of receptor dimerization enables one set of receptor heterodimers (i.e. primary dimers) to give rise to the formation of a new receptor heterodimer complex (designated secondary dimers). Thus, growth factor (EGF, heregulin)-stimulated signaling may actually consist of a defined sequence of cellular activities that are initiated by a specific series of receptor heterodimerization events.

An obvious follow-up question concerns the identity of the specific signaling partners that are recruited by different primary and secondary receptor heterodimer complexes. We initially proposed that c-Src might bind to ErbB3 and participate in signaling pathways involving this protein; however, we have been unable to detect an ErbB3-Src interaction in various cancer cell lines that we have examined. However, we were able to show that the 85 kDa regulatory subunit (p85) of the phosphatidylinositol 3-kinase (PI 3-kinase) did in fact form a stable complex with ErbB3 in a heregulin-dependent manner (32). In fact, it was the characteristics of this specific interaction that provided us with the first clues that receptor heterodimers represented transient rather than stable complexes.

It also was during our efforts to characterize the downstream signaling activities which are stimulated by receptor dimerization events that led us to find c-Cbl as a signaling partner for the EGFR in breast cancer cells. Moreover, we recently have uncovered what appears to represent a novel 'end-point' for growth factor signaling. This was the outcome of our identification of what we initially believed was a novel nuclear GTP-binding activity, which could be strongly activated by heregulin and by other growth factors including nerve growth factor (NGF). Recently, we have determined that this GTP-binding activity is actually part of the RNA cap-binding protein complex (designated CBC) which has been implicated in pre-mRNA splicing and in RNA nuclear export (30,33), thus suggesting that various key aspects of RNA metabolism may be regulated by growth factor-coupled signaling cascades. This in turn raises a number of exciting possibilities regarding how aberrations in RNA splicing, trafficking, and translation may contribute to human cancers.

In the next year, we will continue to study what we feel are new signaling outcomes of EGF- and heregulin-stimulated receptor tyrosine kinase activation. These studies will entail a combination of biochemistry and molecular biology-based approaches. Various cell lines expressing different combinations of the EGFR family will be generated (i.e. COS7 cells expressing EGFR plus Neu, Neu plus ErbB3, Neu plus ErbB4, ErbB3 plus ErbB4) in order to more precisely define what receptor heterodimer combinations are responsible for specific

signaling outcomes. We also are generating breast cancer cell lines that express different forms of (epitope-tagged) Cbl in order to determine exactly how the EGFR mediates the phosphorylation of Cbl and to identify other Cbl-binding partners. In addition, a major emphasis will also be directed toward delineating the signaling pathway that begins with heregulin binding at the cell surface and culminates in the activation of the RNA-binding protein, CBP20, in the nucleus. We will use a dominant-negative Ras mutant to rule out Ras as a participant in this signaling pathway (as has been suggested by our preliminary work thus far) and dominant-active forms of other candidate signal transducers (e.g. Cdc42) to implicate these molecules as upstream activators of CBP20. We also plan to continue our work to establish the fidelity of a canine model system for studying breast cancer.

BODY/"PROGRESS REPORT"

The following is a progress report regarding the work that has been performed by our laboratory during the past year (7/15/96-7/15/97) as funded by the Department of Defense Breast Cancer Initiative USAMRDC grant DAMD17-94-J-4123. This report is subdivided into the following sections, 1.) Description of objectives, 2.) Progress of the research, 3.) Publications and manuscripts resulting from the research (indicated in bold, below), and 4.) Personnel involved in the studies.

1. Description of objectives- The proposal that was submitted to the Department of Defense centered on our finding that the stimulatory effects of heregulin on the Neu tyrosine kinase were mediated through Neu-ErbB3 heterodimer formation (19). [Another group had found that heregulin also binds to ErbB4 and stimulates Neu-ErbB4 heterodimer formation and Neu activation (20)]. Thus, we proposed to verify that Neu-ErbB3 heterodimers in fact form in cells, as indicated by co-precipitation of these receptors using specific anti-Neu and anti-ErbB3 antibodies, and that these interactions led to the (tyrosine) phosphorylation of ErbB3. We were also interested in determining whether other receptor combinations formed heterodimers in cells, since this would point to the possibility that EGF as well as heregulin could activate Neu in breast cancer cells. Finally, we wanted to begin to identify signaling pathways that were initiated by heregulin and/or EGF binding and then to determine whether similar signaling pathways were activated in dog mammary cancer cells.

During the past three years, we have made substantial progress toward achieving many of these objectives. We have shown that heregulin-stimulated Neu-ErbB3 heterodimer formation occurred in cells and that this led to the activation of Neu, the (tyrosine) phosphorylation of ErbB3, and the formation of a complex between phosphorylated ErbB3 and p85. We also showed that other receptor dimer combinations were capable of forming including EGFR-Neu and EGFR-ErbB3 complexes and that each of these heterodimers were capable of initiating distinct signaling pathways (e.g. only the heregulin-stimulated Neu-ErbB3 heterodimer resulted in the formation of a specific complex between ErbB3 and p85). This was further reinforced by our findings that secondary receptor dimers can arise from primary receptor dimerization events (also, see below). As alluded to above, we also identified two potentially new signaling pathways, one that is initiated by EGF and involves the c-Cbl protein and a second that is initiated by heregulin and culminates in the activation of the 18 kDa splicing factor, CBP20. We had indicated in our previous "Progress Report" that we would extend each of these lines of research in the third year of funding. A detailed summary of this work will now be provided. All publications resulting from this work are indicated in bold.

2. Progress of Research:

2a. Identification and characterization of a novel mechanism for growth factor signaling- Our initial work characterizing heregulin-stimulated Neu-ErbB3 heterodimer formation highlighted the transient nature of receptor-receptor interactions and suggested that following trans-phosphorylation, ErbB3 dissociated from Neu and interacted with specific signaling partners [e.g. p85 (32)]. However, an interesting and unanswered question at the time

was what happened to the free activated Neu molecules that were generated by the dissociation of the Neu-ErbB3 dimer? During the past year, we have obtained increasing evidence that the activated Neu molecules are able to associate with (basal) non-activated receptor monomers to form secondary receptor dimers. We have reported two examples of this phenomenon from work performed in rat pheochromocytoma (PC12) cells (**Gamett et al., 1997**). The first example of secondary receptor heterodimer formation was observed upon adding heregulin to PC12 cells. This yielded the expected Neu-ErbB3 primary dimer (and accompanying trans-phosphorylation of ErbB3) but we also detected (heregulin-stimulated) Neu-EGFR complex formation (**see Figure 2 in Gamett et al., 1997**). Since neither Neu nor EGFR binds heregulin, this complex has to arise as an outcome of the primary Neu-ErbB3 heterodimer. The formation of the secondary Neu-EGFR heterodimer was blocked by the addition of a tyrphostin that specifically inhibited Neu tyrosine kinase activity, presumably because the trans-phosphorylation of ErbB3 by Neu is necessary for the dissociation of the primary Neu-ErbB3 heterodimer (which is pre-requisite for the interactions of activated Neu with other receptor monomers to form secondary dimers). We reported a second example of secondary receptor heterodimerization in PC12 cells, which arises from the initial addition of EGF. In this case, EGF-stimulated EGFR-Neu primary heterodimerization gives rise to the trans-phosphorylation and activation of Neu (due to cross-modulation by the EGFR) and the dissociation of the activated Neu molecules. This in turn enables the formation of Neu-ErbB3 secondary dimers (which do not require the addition of heregulin). The extent of Neu-ErbB3 secondary receptor dimerization is markedly reduced by the addition of a tyrphostin that specifically inhibits the EGFR, by blocking the trans-phosphorylation and resultant dissociation of activated Neu from the EGFR. Specific inhibitors of the Neu tyrosine kinase enhance the formation of Neu-ErbB3 secondary dimers by blocking the trans-phosphorylation of ErbB3 by Neu and thereby preventing the breakdown of the secondary dimer.

This work has led us to propose that growth factors can actually stimulate a cascade of receptor dimerization events through a mechanism that reflects the enzyme-substrate nature of the interactions between members of the EGFR family. The trans-phosphorylation of one receptor (i.e. the substrate) by its partner receptor (the enzyme) within a primary receptor heterodimer results in the dissociation of the primary dimer (analogous to the dissociation of an enzyme-product complex) and enables the individual trans-phosphorylated receptors to form new receptor dimers and to phosphorylate new partners. This allows each member of the EGFR family to generate a heterogeneous population of phosphorylated receptor species (depending on the types of primary and secondary receptor dimers that are generated), thereby increasing the diversity of signaling pathways that can be initiated by a single growth factor. In this regard, we have found that the phosphorylation profile for ErbB3 that occurs as an outcome of heregulin-stimulated Neu-ErbB3 heterodimer formation differs from that derived from EGF-stimulated Neu-ErbB3 secondary dimer formation. The signals induced by secondary receptor dimers will likely affect cells that have already been subjected to the first wave of signals emanating from primary receptor dimerization events, such that the second wave of signals might stimulate or arrest, elongate or terminate, the effects induced by the first wave of signals.

2b. Continued studies of growth factor-stimulated signaling through c-Cbl- We have continued to characterize the EGF-dependent tyrosine phosphorylation of an ~130 kDa protein which we identified as c-Cbl. Much of this work is forming the basis of a manuscript that we expect to submit soon (Flanders et al., in preparation). We have examined the phosphorylation of Cbl in a number of different human breast cancer cell lines and have found a strong phosphorylation signal in the following lines, MDA-MB 468, BT10, MDA-MB 231 and SKBR3, all of which show relatively high expression of the EGFR. We have not observed tyrosine phosphorylation of Cbl in MDA-MB 453 cells which lack detectable levels of EGFR. We also have not seen any ability of heregulin to stimulate the phosphorylation of Cbl, which argues for a specific EGFR-mediated signaling event. The finding that c-Cbl is a highly specific phospho-substrate for the EGFR raises some potentially interesting implications, given the likely role for Cbl in signaling processes that impact on cell growth and development (34). The c-Cbl protein contains a number of putative signaling motifs, including a nuclear localization sequence in the

amino-terminal third of the molecule, and a RING finger (zinc-binding motif) and leucine zipper within the carboxyl-terminal two-thirds of the molecule, as well as a number of proline-rich motifs and potential tyrosine phosphorylation sites. The c-Cbl protein becomes transforming after deletion of 17 amino acids carboxyl-terminal to the viral-Cbl coding region (i.e. residues 366-382) (35). This truncated Cbl protein is highly tyrosine phosphorylated, which suggests that the hyper-phosphorylation of Cbl may cause it to become over-active in some type of signaling pathway, thereby triggering transformation.

An important priority has been to begin to delineate the signaling pathways emanating from the EGF-stimulated phosphorylation of Cbl, which may contribute to transforming signals. Toward this end, while searching for binding partners for c-Cbl in human breast cancer cell lines, we found that the c-Crk adaptor molecule associated with phosphorylated Cbl in an EGF-dependent manner. The Cbl-Crk interaction appears to be mediated by the binding of the SH2 domain (and not the SH3 domain) of Crk to a phosphorylated tyrosine residue on Cbl. This interaction is observed in all breast cancer cell lines that show the EGF-stimulated phosphorylation of Cbl and the time course for the EGF-stimulated phosphorylation exactly matches the time course for the Cbl-Crk interaction.

The obviously important question concerns whether the formation of an EGF-stimulated Cbl-Crk complex has important signaling implications. It has been shown that Cbl interacts with p85 in an EGF-dependent manner (36,37) and it has been suggested that Cbl may serve as an interface or an adaptor molecule for EGFR effects on PI 3-kinase activity in human epidermoid carcinoma (A431) cells (38). While this is an interesting possibility, as yet we do not know exactly how the EGFR couples to Cbl. We have not been able to demonstrate a direct interaction between the EGFR and Cbl, by immunoprecipitation (this may mean that the EGFR-Cbl interaction is relatively weak), although, we have found that Cbl will bind to the amino-terminal SH3 domain of Grb2, suggesting that this adaptor may serve to interface the EGFR with Cbl. Nonetheless, it is possible that Cbl, in turn, serves to interface the EGFR with Crk and Crk-binding proteins (e.g. the Abl tyrosine kinase) in mammary cells. This possibility is particularly intriguing given that it has been proposed that aberrant tyrosine phosphorylation of Cbl by Abl may contribute to its transforming activity (35) and that transformation by oncogenic Crk (i.e. following the deletion of its carboxyl-terminal SH3 domain) may be due to the excess tyrosine phosphorylation mediated by the interaction between oncogenic Crk and Abl (39).

However, another interesting possibility is that c-Cbl may play a role in signaling pathways that regulate the guanine nucleotide activity of small G proteins. In activated Jurkat T cells, the c-Crk related adaptor protein, CrkL, becomes associated with tyrosine phosphorylated c-Cbl (40). CrkL was constitutively associated with the guanine nucleotide exchange factor, C3G, suggesting a possible mechanism by which Cbl may influence the regulation of Ras and/or Ras-subfamily proteins. Moreover, recently we have found that a novel member of the Dbl family of Rho-guanine nucleotide exchange factors, designated Cool (for cloned-out-of a library), binds to the c-Cbl homolog, Cbl-b (41). A proline-rich domain on Cbl-b binds to a SH3 domain on Cool, which is interesting because Cbl and Cbl-b are predicted to have similar SH3-binding capabilities. At present, we do not know the physiological function of Cool, nor the ramifications of a Cbl- Cool interaction. However, we originally identified Cool through its ability to bind to a serine/threonine kinase called PAK (for p21-activated serine/threonine kinase), which is a target for the Cdc42 and Rac GTP-binding proteins. The Dbl-homology domain of Cool would also predict that it binds Cdc42 or Rac. Thus, Cool may provide another mechanism by which Cbl might influence the regulation/activation of small GTP-binding proteins.

2c. Further connections between growth factor signaling and Rho-like GTP-binding proteins- We have been searching for possible connections between Cdc42 and cell growth regulation because of the potential involvement of Cdc42 or related Rho-like small G proteins in Cbl-mediated signaling, as well as their possible involvement in a specific heregulin-stimulated pathway that culminates in the activation of a putative splicing factor. An essential role for Cdc42 in cell growth regulation has been suggested by the finding that the Dbl oncoprotein is an upstream activator (i.e. a guanine nucleotide exchange factor) for Cdc42 (**see Zheng et al.,**

1996) and that other Dbl-related proteins, which cause cellular transformation, are nucleotide exchange factors for Rho proteins (**see Glaven et al., 1996**). Because we have suspected that activation of Cdc42 will stimulate cell growth, we have examined the effects of dominant-active, GTPase-defective Cdc42 mutants in different cell lines. However, we have typically found that over-expression of GTPase-defective Cdc42 mutants gives rise to growth inhibition. This has led us to hypothesize that the ability of Cdc42 to undergo a complete GTP-binding/GTPase cycle may be critical for a positive growth signal. In this case, the ability of Cdc42 to bind GTP would be essential for its interactions with downstream target/effectors (as is typically the case for G proteins), but the release of one or more target/effectors may ultimately be necessary for a positive growth signal. Given this suggestion, we have set out to identify Cdc42 mutants that more closely mimic the functional effects of Dbl, that is mutants that are able to spontaneously undergo GTP-GDP exchange while still being capable of GTP hydrolysis. We recently have identified such a mutant by substituting a leucine residue at position 28 for the normal phenylalanine residue (**Lin et al., submitted**). This Cdc42 mutant [Cdc42(F28L)] is able to rapidly exchange GTP for GDP and still hydrolyze GTP. When introduced into cultured fibroblasts, Cdc42(F28L) activated the c-Jun kinase (JNK1), and stimulated filopodia formation, which are hall-marks for the *in vivo* activation of Cdc42. NIH3T3 cells stably transfected with Cdc42(F28L) also showed reduced contact inhibition, lower dependence on serum for growth, and anchorage-independent growth, all characteristics that are exhibited by oncogenic Dbl. Recent findings have also shown that activated Cdc42 is necessary for Ras-induced transformation. Thus, these studies all point to the possibility of Cdc42 being an important downstream signaling transducer in growth factor-coupled mitogenic pathways.

This, in turn, has led us to try to identify and characterize Cdc42-targets that might participate in growth factor-coupled signaling. One particularly interesting possibility is a novel non-receptor tyrosine kinase that we recently identified and shown to be an absolutely specific binding-partner for Cdc42 (and not for Ras or Rho) (**Yang and Cerione, submitted**). This tyrosine kinase is highly related to ACK (for activated Cdc42-kinase) and thus we have designated it as ACK-2. Over-expression of ACK-2 in COS cells yields an apparently constitutively active tyrosine kinase. However, when the cells are detached (i.e. by trypsin treatment), the tyrosine kinase activity of ACK-2 is severely depressed. The cell attachment-dependent activation of ACK-2 appears to require activation of Cdc42; the expression of dominant-active (GTPase-defective) Cdc42 accentuates the ACK-2 activation that occurs due to cell attachment, whereas expression of dominant-negative Cdc42 prevents ACK-2 activation. When cells are detached, the ACK-2 activity can then be stimulated by the addition of serum or by the addition of EGF. This then suggests that ACK-2 represents a possible point of convergence between signaling pathways that are initiated by EGFR family members and those that involve (activated) Cdc42. We plan to examine the possibility that Cdc42 and/or ACK-2 participate in EGF-stimulated signaling to c-Cbl, and in particular, if Cdc42 is recruited to Cbl by Cool and if this in turns leads to the recruitment of ACK-2 and the resultant tyrosine phosphorylation of Cbl.

2d. Identification of a novel heregulin-stimulated GTP-binding activity as the RNA splicing factor, CBP20- During the past year, we have been characterizing a GTP-binding activity that is present in nuclear extracts in every cell type that we have examined and is strongly stimulated by heregulin (Wilson et al., in preparation). We initially showed that in HeLa cells this activity (as assayed by the photo-incorporation of [α^{32}P]GTP) was most effectively stimulated by heregulin, whereas in rat PC12 cells, the activity was also strongly stimulated by nerve growth factor (NGF) (see Figure 1 at the end of the 'Progress Report'). This finding was consistent with earlier work from our laboratory (42), which indicated that heregulin, like NGF, was capable of stimulating neurite extension in PC12 cells. The heregulin/NGF-stimulated guanine nucleotide-binding activity corresponded to a protein of apparent M_r ~ 18 kDa, which we initially designated as p18. The incorporation of [α^{32}P]GTP into p18 was also catalyzed by the exposure of cells to ultraviolet radiation (Figure 2), suggesting a stress response similar to those observed with the nuclear MAP kinases, the c-Jun kinase (JNK1) and p38. The growth factor-stimulated binding of GTP to p18 also showed a cell-cycle dependence, such that it was strongest

in G_1/S and not detected in G_0 nor when cells were blocked in mitosis (Figure 3). Perhaps most interesting, we have found that p18 activity is strongly activated in the human breast cancer cell line, SKBR3 (Figure 1A), even under conditions of serum-starvation. At the present time, we know very little about the detailed sequence of events that lead to the activation of p18. However, as will be elaborated upon below, this becomes an especially interesting issue because we have recently determined that p18 is identical to CBP20, an 18 kDa subunit of the nuclear RNA cap-binding protein complex [CBC (30)].

During our attempts to purify p18, we found that it eluted from gel filtration columns with an apparent size of ~150 kDa and appeared to co-purify with an ~80 kDa protein (designated p80), which we suspected might be essential for the GTP-binding activity of p18. The nuclear localization of p18 and its apparent requirement of p80 for binding guanine nucleotides were highly reminiscent of the biochemical properties of the RNA cap-binding complex (designated CBC), which has been implicated in precursor mRNA splicing and in RNA nuclear export (30,33). This complex is comprised of an 18 kDa protein (CBP20) and an 80 kDa protein (CBP80), which when associated are able to bind to a 5' cap structure that consists of a guanosine residue methylated at the N7 position and joined to the first encoded nucleotide of RNA. The first experimental indication that p18 and p80 may correspond to CBP20 and CBP80, respectively, came from Western blot analyses of the purified fractions of p18, which showed that these fractions were highly enriched in both the CBP20 and CBP80 proteins. We then found that p18 was capable of binding RNA-cap analogs even more effectively than GTP and that p18/GTP-binding activity could be immunoprecipitated from cells using an anti-CBP80 antibody (Figure 4). We also have found that *E. coli*-expressed CBP20 can be radiolabeled with [α^{32}P]GTP in a CBP80-dependent manner, and we have demonstrated serum-starved GTP-binding to recombinant CBP20 that was expressed in BHK21 cells. Based on these findings, we have concluded that p18 is identical to CBP20.

The stimulation by heregulin of CBP20 binding to RNA cap structures may have important implications regarding a previously unappreciated connection between receptor tyrosine kinase (i.e. Neu) signaling and RNA splicing, RNA export and/or protein synthesis. It also raises the possibility of some potentially interesting growth factor-regulated interactions between CBP20 and other proteins that have been implicated in RNA metabolism. Of particular interest is the GTP-binding protein Ran, which has been implicated in RNA trafficking and cell-cycle control, and its activator [guanine nucleotide exchange factor (GEF)] RCC1 (43,44). Studies with tsBN2 cells which possess a temperature-sensitive RCC1 protein have shown that the loss of RCC1 at the non-permissive temperature is accompanied by a marked increase in the ability of CBP20 to be labeled with [α^{32}P]GTP. One possible explanation for these findings is that RCC1 serves as a guanine nucleotide-dissociation inhibitor (GDI) for CBP20, such that when CBP20 is associated with RCC1, GDP (or GTP) dissociation from CBP20 is blocked and the incorporation of radiolabeled GTP is inhibited. Thus, a growth factor (heregulin)-stimulated signaling pathway to the nucleus may in some manner disrupt the interaction between CBP20 and RCC1, thus enabling CBP20 to undergo guanine nucleotide exchange or the exchange of bound guanine nucleotide for capped RNA.

CONCLUSIONS

1. Significance of the Research- It is generally agreed that aberrations in the signaling pathways emanating from receptor tyrosine kinases will have severe consequences in terms of the development of human cancer. This appears to be especially the case for breast cancer, where some strong correlations exist between the expression of the Neu tyrosine kinase and the prognosis for patients with this disease. The observations that other members of the EGFR family are also over-expressed in certain breast cancer cell lines, further argues for the potential involvement of these receptor tyrosine kinases in the development of mammary carcinomas. During the past year, we have continued to make good progress on studies of how these receptor tyrosine kinases signal, as laid out in the original goals of our proposal to the Department of Defense. We have uncovered a new mechanistic aspect of receptor-receptor interactions and have shown that

members of the EGFR family form heterodimers that are transient in nature. Thus, Neu-ErbB3 heterodimers can form as primary dimers, stimulated by the initial binding of heregulin to ErbB3, or as secondary dimers as an outcome of the dissociation of activated Neu from EGF-promoted EGFR-Neu primary dimers. Interestingly, it appears that the phosphorylation profiles for ErbB3 which arise from these two types of Neu-ErbB3 heterodimers are not identical. This then argues that the ability of different growth factors to stimulate the formation of both primary and secondary receptor dimer combinations adds to the diversity of signaling activities that can be generated by the EGFR family.

We also have continued to obtain new insights into two novel signaling pathways. One involves the specific tyrosine phosphorylation of c-Cbl by the EGFR in breast cancer cells. While we still do not know the ultimate biological consequences of this phosphorylation event, we are beginning to examine the possibility that Cbl may provide an important interface between the EGFR and signaling that is mediated by the Ras-related protein Cdc42 (and/or the related G protein, Rac). We have shown that activators (guanine nucleotide exchange factors) for Cdc42 and related G proteins are often oncogenic and we recently have discovered that specific mutations of Cdc42 cause it to become potently transforming. We also have discovered a new putative adaptor/regulator for Cdc42, called Cool, which may directly bind Cbl, and a new tyrosine kinase that serves as a potential target/effector for Cdc42 action. Thus, we are excited about the possibility that Cbl serves as a scaffold for interfacing regulators and targets for Cdc42 with the EGFR.

A second novel signaling end-point for growth factor action which we have uncovered is an ~18 kDa protein which appears to be essential for the normal regulation of RNA splicing. We initially discovered this protein while searching for new types of nuclear GTP-binding activities; however, after purification efforts, we determined that this GTP-binding activity was identical to the CBP20 protein, which normally binds the ^{7}methyl guanosine cap structure of precursor RNA molecules that are transcribed by RNA polymerase II. Since CBP20 activity appears to be strongly regulated by heregulin, we now are positioned to obtain new information regarding how Neu and/or related receptor tyrosine kinases influence different aspects of RNA metabolism. Because approximately 15% of all genetic diseases have been attributed to alterations in RNA splicing (45), and because there have been a number of correlations drawn between the generation of improper RNA transcripts and tumorigenesis (46,47), these findings hold exciting potential for providing some novel insights into how growth factors normally influence RNA processing at the level of gene expression and how a loss of this regulation may have important implications for the development of various diseased states including cancer.

2. Methods of Approach: Plans for the Coming Year- During the coming year, we will continue our efforts on studying each of the above described aspects of growth factor receptor-coupled signaling. We also will determine whether these signaling activities show the same characteristics in canine mammary carcinomas, which we are trying to establish as a model for human breast cancer. These studies will be pursued through the following specific objectives.

a.) Do primary and secondary receptor heterodimers initiate distinct signaling pathways? Now that we have established that (primary) receptor heterodimers can dissociate following receptor activation and trans-phosphorylation and form new dimer combinations (i.e. secondary dimers), a critical question concerns whether a particular dimer combination initiates distinct signaling events, depending on whether it is a primary or secondary dimerization event. We are particularly interested in this question for Neu-ErbB3 primary and secondary dimers, since the primary dimers are initiated by heregulin binding to ErbB3 while the secondary dimers are an outcome of EGF-stimulated EGFR-Neu interactions. There has been a good deal of interest in understanding the biological consequences of Neu activation via EGFR trans-modulation versus heregulin-stimulated ErbB3-Neu interactions. Thus, we will set out to determine whether primary Neu-ErbB3 dimers recruit different cellular signaling molecules versus Neu-ErbB3 secondary dimers. One example is the 85 kDa regulatory subunit of the PI 3-kinase, which we have shown to form a specific complex with ErbB3 in a heregulin-dependent manner. It will be interesting to see if in fact this complex does not occur as an outcome of (EGF-stimulated) Neu-ErbB3

secondary dimer formation. We also will examine the possibility that c-Src binds to ErbB3 following Neu-ErbB3 secondary dimerization. We had proposed to look for ErbB3-Src interactions in our original proposal, however, we were not able to obtain evidence for such interactions in breast cancer cells. It is interesting to speculate that those interactions may not occur following Neu-ErbB3 primary dimerization, but only when Neu trans-phosphorylates ErbB3 within a secondary dimer.

b.) Does Cbl serve as a signaling scaffold to interface the EGFR with Cdc42 in breast cancer cells? We have recently found that a Cdc42-binding protein, which we have designated Cool, interacts with members of the Cbl family (based on yeast 2-hybrid studies). Cool also interacts with and appears to negatively regulate PAK (for p21-activated kinase) which is a target for the Cdc42 and Rac GTP-binding proteins. We have proposed that Cool and PAK are two Cdc42-targets that need to be coordinately regulated, i.e. Cdc42 initially binds and activates PAK but then upon the binding of activated Cdc42 to Cool, the serine/threonine kinase activity of PAK is attenuated (through Cool-PAK interactions) and a specific Cool-mediated signaling pathway is engaged. The ability of Cool to bind Cbl suggests a potential mechanism by which EGFR signaling may influence PAK activity (which normally sends signals to stress-activated nuclear MAP kinases) and initiate a distinct Cdc42 signaling event. We will set out to establish whether this in fact is the case by determining whether EGF promotes the interactions between Cbl and Cool and/or between Cdc42 and Cool. We also will determine whether the EGFR promotes the activation of Cdc42 in the same breast cancer cells that we find EGF-stimulated (tyrosine) phosphorylation of Cool. Finally, we will determine whether a new tyrosine kinase target for Cdc42, called Ack-2, is capable of binding and/or phosphorylating Cbl in different breast cancer cell lines.

c.) Continued studies of heregulin-mediated regulation of the RNA splicing factor, CBP20. A good deal of emphasis during the coming year will be placed on understanding how heregulin influences the activity of CBP20. We first intend to delineate the signaling pathway that normally mediates the actions of heregulin on this splicing factor. Is it a heregulin-stimulated (primary) heterodimerization between ErbB3 and Neu that culminates in the activation of CBP20 and/or do other EGFR family members contribute to the regulation of CBP20? Is the signaling mediated through the activation of Ras or do other Ras-related proteins play a critical role (such as Cdc42)? We also will set out to determine exactly how CBP20 activity is regulated in the nucleus. Is the GTP-binding activity that we have identified an important component of the nuclear regulation? For example, does CBP20 normally contain bound GTP and needs to await a growth factor-coupled signal to initiate the exchange of GTP for capped RNA? Or, does the GTP-binding activity of CBP20 serve another purpose, i.e. by mediating the regulation of another nuclear activity or nucleocytoplasmic transport event? Finally, we will examine whether heregulin influences the interactions of CBP20 with other nuclear proteins suspected to play a role in RNA metabolism, of particular interest being the Ran GTP-binding protein and its upstream activator, RCC1.

d.) Establishment of an animal model system for human breast cancer. During the coming year, we will continue our efforts to establish the fidelity of an animal model system for human breast cancer, which is based on the development of spontaneous mammary carcinomas in the dog. Our previous efforts have suggested that Neu is over-expressed in dog mammary tumors (we are still determining the exact percentage) and that EGF-stimulates the specific tyrosine phosphorylation of c-Cbl and its interaction with c-Crk, just as has been observed in human breast cancer cell lines. We now plan to determine whether CBP20 is constitutively active in dog mammary carcinomas that over-express ErbB2, just as we have found in the human breast cancer cell line, SKBR3. We also will determine whether other aspects of heregulin-promoted activation/regulation of CBP20 occur in dog mammary cells in a manner identical to what has been shown in human mammary cells. The ultimate hope is that the activation of CBP20 may prove to be a strong criterion for the usefulness of this animal model and possibly serve as a sensitive indicator for the onset of the disease. The longer term goal is to be able to examine how small molecules that influence CBP20 activity affect dogs that have been diagnosed with mammary tumors.

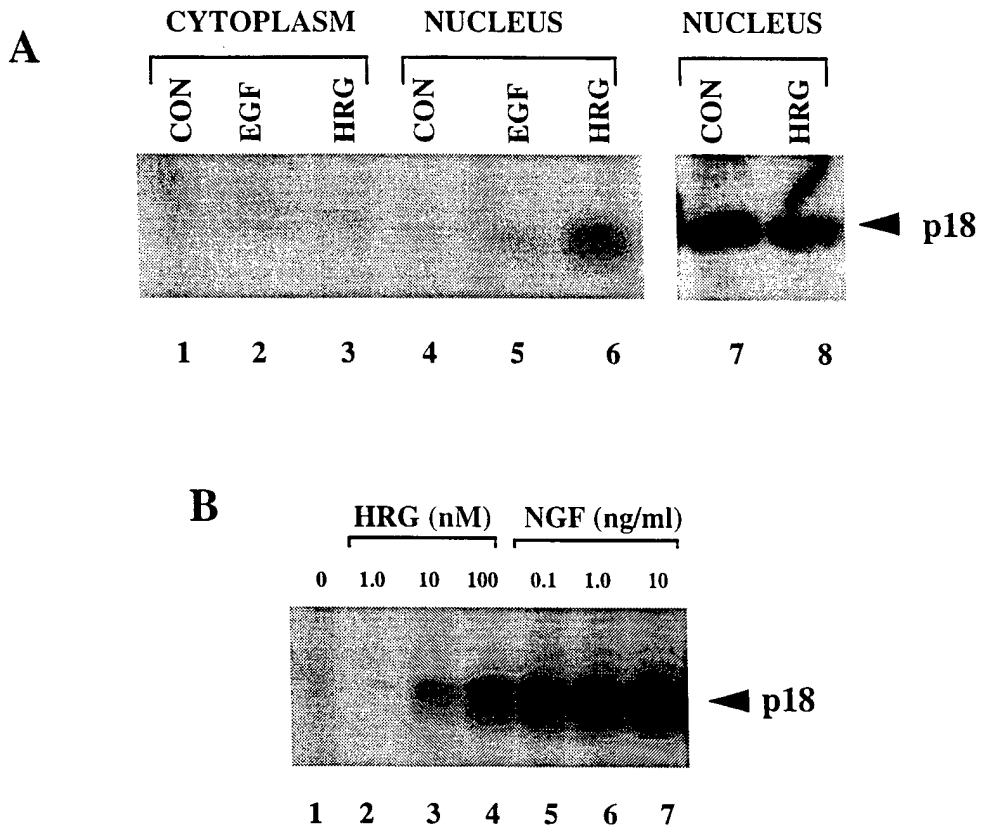


FIG. 1. **Growth factor sensitivity of an 18 kDa nuclear GTP-binding activity in HeLa cells, PC-12 cells, and the breast cancer cell line, SKBR3.** A) HeLa cells (*lanes 1-6*) were serum starved (*control lanes 1 and 4*) and then treated with 100 ng/ml EGF (*lanes 2,5*), or with 30 nM heregulin (HRG, *lanes 3,6*) for 15 minutes at 37°C. SKBR3 cells were serum starved (*lane 7*) and then treated with 1.0 nM heregulin for 1.5 hours (*lane 8*). The cells were then lysed and separated into cytoplasmic (*lanes 1-3, 7*), and whole nuclear (*lanes 4-6, 8*) fractions and assayed for GTP-binding using 45 µg of HeLa lysate or 50 µg of SKBR3 lysate. The samples were incubated for 10 minutes at room temperature with 20 µl of crosslinking buffer and 3 µCi [α^{32}P]GTP, followed by exposure to UV light for 15 minutes on ice. The samples were then separated by 15% SDS-PAGE, and the resulting gel was dried and exposed to X-ray film for 11 h to 3 days. B) A dose response experiment was performed using the addition of either heregulin (*lanes 2-4*) or NGF (*lanes 5-7*) to serum-starved PC-12 cells (*control, lane 1*) for 30 minutes at 37°C. For each dose of heregulin or NGF, 50 µg of total nuclear lysate protein were assayed for GTP-binding by UV-crosslinking with [α^{32}P]GTP, and then separated by 15% SDS-PAGE. The resulting gel was dried and exposed to X-ray film for 5-15 h.

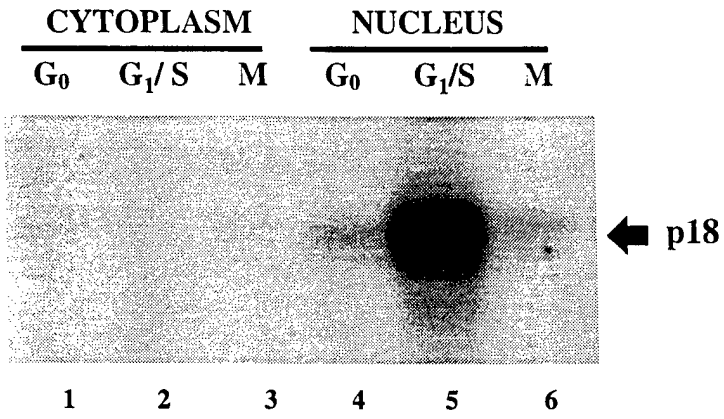


FIG. 2. **p18 is active to bind GTP in cells arrested in G_1/S phase of the cell cycle.** HeLa cells were arrested in G_0 by serum starvation (*lanes 1,4*), in G_1/S by 2.5 mM thymidine addition (*lanes 2,5*), and in M phase with 80 ng/ml nocodazole (*lanes 3,6*). Cells were then separated into cytoplasmic (*lanes 1,2,3*), and whole nuclear fractions (or a mitotic pellet was prepared for M phase arrest) (*lanes 4,5,6*), and then for each fraction, 50 µg of protein were assayed for [α^{32}P]GTP-binding activity by UV-crosslinking followed by 15% SDS-PAGE and autoradiography.

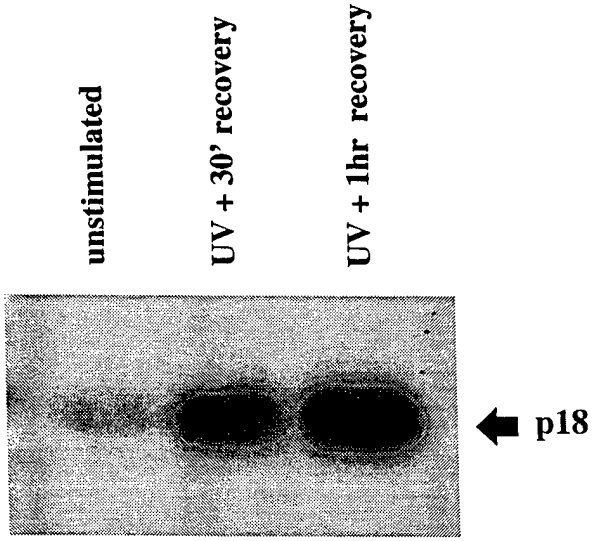
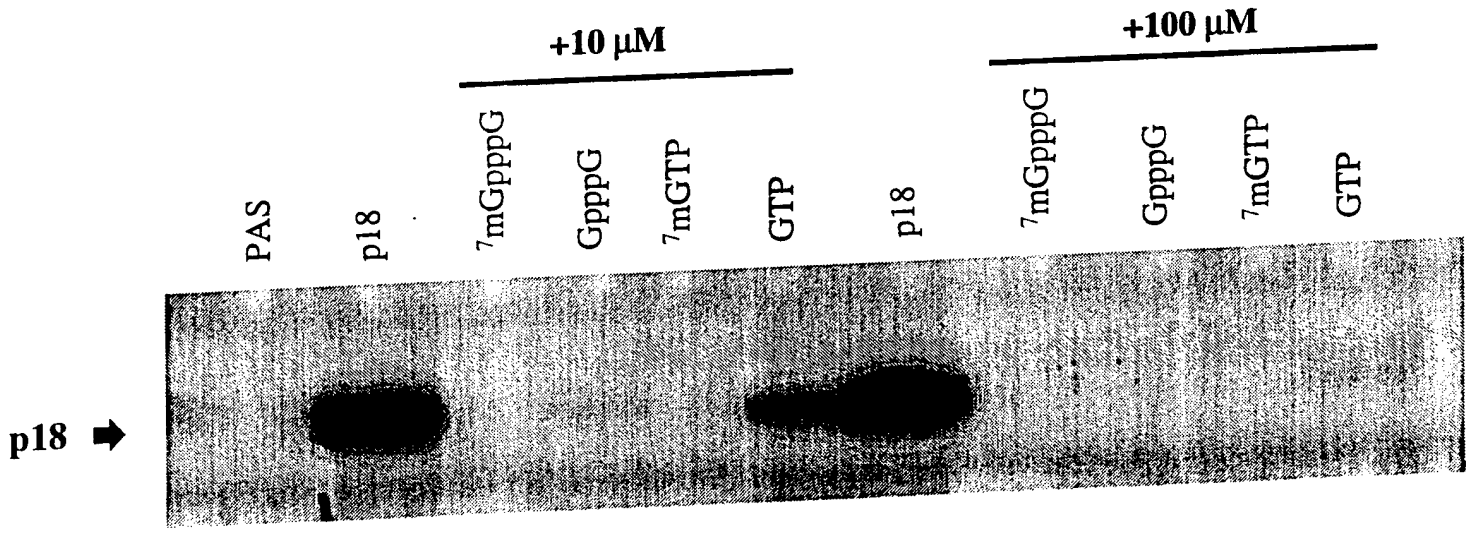


FIG. 3. **Exposure of cells to UV light stimulates the GTP-binding activity of p18.** PC-12 cells were serum-starved and then exposed to UV light for 2 minutes. Following exposure, cells were replenished with serum-free medium and allowed to recover for 30 minutes or one hour. Cells were then harvested, nuclear lysates were prepared, and 50 µg of nuclear lysate were assayed for [α^{32}P]GTP-binding to p18 by UV crosslinking followed by 15% SDS-PAGE and autoradiography.



IP: αCBP80
Assay: [α^{32}P]GTP-binding

FIG.4. **p18 [α^{32}P]GTP-binding activity co-immunoprecipitates with CBP80 from PC-12 cells and is blocked by the addition of RNA cap-analogs.** Nuclear extracts were prepared from PC-12 cells growing asynchronously in culture. Five hundred micrograms of lysate were then immunoprecipitated with 5 μl of αCBP80 antiserum and 40 μl of protein A sepharose per lane. The immunoprecipitates were then washed four times with Tris-HCl, pH 7.4, 0.33% (v/v) Triton X-100, 133 mM KCl, 1 mM DTT, and 1 mM Na_3VO_4 and then resuspended in 30 μl of UV-crosslinking buffer. The immunoprecipitates were then assayed for [α^{32}P]GTP-binding to p18 by UV crosslinking in the presence of 10 μM or 100 μM of the cap analogs ^{7}mGpppG, GpppG, ^{7}mGTP, or GTP. These proteins were then separated by 15% SDS-PAGE, and the resulting gel was dried and autoradiogrammed.

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PUBLICATIONS/MANUSCRIPTS RESULTING FROM THE RESEARCH PERFORMED DURING THE PERIOD 7/15/96-7/15/97

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7. Wilson, K.F., Singh, U.S., and Cerione, R.A. Identification of the RNA-cap binding protein, CBP20, as a novel nuclear target for growth factor-coupled signal transduction. in preparation.
8. Flanders, J.A., Thompson, A.M., Knudsen, B., and Cerione, R.A. The c-Cbl proto-oncogene product is a highly specific signaling partner for the EGF receptor in human breast cancer cells. in preparation.

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Secondary Dimerization between Members of the Epidermal Growth Factor Receptor Family*

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Growth factor receptors of the epidermal growth factor (EGF) receptor family play pivotal roles in the regulation of cell proliferation and differentiation and are involved in the development of human cancers. It has been well documented that these receptors undergo growth factor-stimulated homo- and heterodimerization as a first step in the initiation of signaling cascades. Here we provide evidence for a new mechanism for growth factor-stimulated receptor dimer formation, designated secondary dimerization. The growth factor-induced dimerization and ensuing receptor trans-autophosphorylation results in the dissociation of the original (primary) receptor dimer. Each phosphorylated receptor monomer then interacts with a new (nonphosphorylated) receptor to form a secondary dimer. Treatment of cells with EGF yields Neu-ErbB3 secondary dimers, and heregulin treatment induces the formation of Neu-EGF receptor (secondary) dimers. The ability of EGF and heregulin to stimulate a cascade of dimerization events points to a novel mechanism by which multiple signaling activities and diverse biological responses are initiated by members of the EGF receptor family.

The interactions of various growth factors and cytokines with cell surface receptor tyrosine kinases initiate a variety of intracellular signaling pathways that when integrated yield cell cycle progression, cell differentiation, or apoptosis. Several classes of receptor tyrosine kinases have been described, among which the EGFR family is of particular interest, because these receptors have been implicated in malignant transformation (1–8). This family includes the epidermal growth factor (EGF)[1] receptor (also referred to as ErbB1), the Neu/ErbB2 protein (referred to from here on as Neu), and the more recently identified ErbB3 and ErbB4 proteins (9–12). Two types of ligands interact with members of the EGFR family; EGF is a prototype for ligands that bind to the EGFR, and heregulin (HRG) or Neu differentiation factor represents a family of ligands that bind to both ErbB3 and ErbB4 (10, 13–15). Two of the receptors, EGFR and ErbB4, are capable of ligand-stimulated tyrosine kinase activity. Thus far, the Neu tyrosine kinase is an orphan receptor, whereas ErbB3 appears to be a kinase-defective receptor (14, 16).

The binding of EGF or HRG to their receptors results in receptor dimerization and receptor trans-autophosphorylation. The phosphorylated receptors recruit cellular signaling proteins, through the binding of their Src homology 2 or phosphotyrosine binding domains and thus initiate signaling pathways (17, 18). The binding of EGF stimulates the formation of both EGFR homodimers or heterodimers like EGFR-Neu (19–21). Similarly, HRG can stimulate receptor homodimer formation of ErbB3 or of ErbB4 as well as receptor heterodimers like ErbB3 and Neu or ErbB4 and Neu (13, 15). The HRG-promoted formation of these heterodimers provides the molecular basis for the stimulation of Neu tyrosine kinase activity (7, 13, 15, 22).

The mode of heterodimerization between members of the EGFR family may have a significant influence on malignant transformation. For example, the co-expression of ErbB3 and Neu in NIH3T3 cells results in neoplastic transformation, whereas neither the expression of ErbB3 nor Neu alone is sufficient for transformation (1, 7). The heterodimerization of the EGFR with Neu results in cell transformation, whereas the replacement of wild type Neu by its kinase-defective counterpart abrogates transformation (2, 23). Moreover, the expression of Neu in breast and ovarian carcinomas correlates with a poor prognosis (1, 2, 5, 6). The molecular basis for this is likely due to an enhanced tendency of the orphan Neu receptor to form heterodimers with other members of the EGFR family (16, 22, 24).

In this study we present evidence for a new mode of inter-receptor interaction, designated secondary dimerization. Our results show that ligand-induced dimer formation might be followed by dimer dissociation and interaction of the individual, activated receptors with inactive receptors to form new, secondary dimers.

MATERIALS AND METHODS

Cells, Growth Factors, Antibodies, and Inhibitors—Rat pheochromocytoma PC12 cell line I77.2 (25, 26) was used in this study. Recombinant heregulin (rHRG-$\beta1_{177-241}$) was provided by Dr. Mark Sliwkowski (Genetech, Inc.), and EGF was purchased from Sigma. Anti-EGFR polyclonal antibodies (SC 03) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-c-Neu monoclonal antibodies (Ab-4) were from Calbiochem (San Diego, CA), anti-ErbB3 (2F12) and anti-c-ErbB4 (AB-2) monoclonal antibodies were from Neo Markers (Fremont, CA), and anti-phosphotyrosine monoclonal antibodies (PY20) were from Transduction Laboratories (Lexington, KY). Antibodies were tested for their specificity, and no cross-reactivity was detected. Tyrphostins were purchased from Calbiochem (San Diego, CA).

Cell Growth, Immunoprecipitation, Western Blots, and Determination of Tyrosine Kinase Activity—PC12 cells were grown on 15-cm plastic dishes in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 units/ml of penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Amphotericin B (Sigma) in a CO_2 incubator (5% CO_2) at 37 °C.

Cell stimulation, immunoprecipitation, and Western blotting were carried out as described previously (26). Nearly confluent cell cultures were incubated for 16–20 h in growth medium containing 0.1% serum. Then the cells were gently detached by a short incubation with Hanks'

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[1] The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; HRG, heregulin.

Appendix 1

balanced salt solution (without calcium or magnesium), centrifuged at 500 × *g* for 5 min, and resuspended in serum-free growth medium. The use of cell suspensions enables the stimulation of a large number of cells in each sample. In control experiments, we did not find a difference between the results obtained when using either attached cells or suspended cells.[2] Cells were dispensed at 1.0×10^7 cells/1.5 ml in Eppendorf tubes, and HRG or EGF (20 nM or 100 ng/ml, respectively) was added to some of the tubes for 2 min at 37 °C. Cells subjected to treatment with tyrosine kinase inhibitors were preincubated for 2 min at 37 °C with tyrphostins before the addition of the growth factors. Tyrphostins AG556, AG879, and AG1478 were added to final concentrations of 25, 25, and 0.5 μM, respectively. The incubations were terminated by transferring the tubes to ice, followed by immediate centrifugation to pellet the cells and removal of the medium. The cell pellets were treated with cold lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidat P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotonin, 1 μg/ml leupeptin, 1 mM EGTA, 40 mM sodium fluoride, 0.1 mM ammonium molybdate, and 1 mM sodium orthovanadate. After 15 min of incubation on ice, the cell lysates were centrifuged for 10 min at 15,000 × *g* to remove insoluble material. Antibodies were then added to the lysates, as needed for each experiment, followed by 30 μl of a 50% suspension of protein A-Sepharose (Sigma). The tubes were incubated for 3 h in the cold with slow agitation, and then the immunocomplexes were pelleted by centrifugation, washed once with lysis buffer, and boiled for 3 min in 40 μl of Laemmli sample buffer. The samples were subjected to SDS-polyacrylamide gel electrophoresis (8% acrylamide), and electrophoretically transferred to polyvinilidene difluoride membranes (Immobilon-P, Millipore, Burlington, MA). The blots were blocked by 1 h of incubation with 3% bovine serum albumin in Tris-buffered saline with 0.05% Tween 20, washed, and incubated for 1 h with primary antibodies as indicated. Blots were analyzed by chemiluminescence using horseradish peroxidase-conjugated secondary antibodies and ECL detection reagents (Amersham Corp.).

Determination of tyrosine kinase activity in the immunoprecipitates was performed as described previously (27). Cell growth, cell exposure to either HRG or EGF, cell lysis, and immunoprecipitation were carried out as described above for immunoblotting. Each immunoprecipitate was washed twice with a buffer containing 10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.1 mM sodium vanadate and resuspended in 60 μl of the same buffer. The assay was initiated by the addition of 20 μl of immunoprecipitate to 20 μl of reaction mixture containing 10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.1 mM sodium vanadate, 10 μM ATP, 2 μCi of [γ-^{32}P]ATP, and 0.5 mg/ml poly-Glu_4-Tyr_1. Some of the reaction mixtures contained tyrphostins, either AG879, AG556, or AG1478, at the respective concentrations of 50, 50, and 1 μM. The assays were conducted for 10 min at room temperature and were terminated by the addition of 10 μl of 0.5 M EDTA. The tubes were centrifuged, and the supernatants were transferred to Whatman 3MM paper strips, which were incubated overnight in 10% trichloroacetic acid, containing 10 mM sodium pyrophosphate at 4 °C, with gentle agitation. The paper strips were then dried and counted in a scintillation counter.

Assays of tyrosine kinase activity of receptors expressed in insect cells were carried out as described previously (28, 29). Briefly, membranes prepared from insect cells (Sf21) preinfected with recombinant viruses (28) harboring either *erb1*, *neu*, or *erbB4* (the latter was obtained from K. L. Carraway (Department of Cell Biology Harvard Medical School, Boston, MA)) were incubated for 10 min at room temperature in a 40-μl reaction mixture containing 20 mM HEPES buffer, pH 7.4, 5 mM $MnCl_2$, 0.5 mM sodium vanadate, 0.5 mg/ml poly-Glu_4-Tyr_1, and 5 μM of [γ-^{32}P]ATP (10 Ci/mmol). The reaction was terminated by the addition of 10 μl of 5 × Laemmli sample buffer (30), and samples were subjected to SDS-polyacrylamide gel electrophoresis (8% acrylamide). Bands of interest were excised from the dried gels, and the associated radioactivity was counted in a scintillation counter (28, 29). Receptors were expressed in insect cells infected with viruses harboring the receptor genes but not in noninfected cells or in cells infected with the wild type virus, as was determined by Western blots.

RESULTS AND DISCUSSION

Indications for a new mode of inter-receptor interactions between members of the EGFR family were obtained during a study of the effects of tyrphostins, specific inhibitors of tyrosine kinase activity (31), on receptor dimerization and tyrosine kinase activity in PC12 cells. In these experiments, cells were exposed to EGF or HRG and then subjected to lysis and immunoprecipitation by anti-EGFR or anti-Neu antibodies. The tyrosine kinase activity in the immunoprecipitates was inhibited by the tyrphostin AG556, a specific inhibitor of EGFR, and by AG879, which specifically inhibits Neu in various cells (27, 31–33), including PC12 cells (34).

TABLE I
Effect of tyrphostins on tyrosine kinase activity

PC12 cells were treated with a ligand and lysed, and receptors were immunoprecipitated. Tyrosine kinase activity was determined in the immunoprecipitates as described under "Materials and Methods" and in Ref. 27.

Ligand	IP[a]	Tyrphostin	TK[b]
EGF	Anti-EGFR	AG879	0.50 ± 0.22
		AG556	0.39 ± 0.15
EGF	Anti-Neu	AG879	0.56 ± 0.15
		AG556	0.53 ± 0.19
HRG	Anti-Neu	AG879	0.52 ± 0.16
		AG556	0.30 ± 0.02
HRG	Anti-EGFR	AG879	0.89 ± 0.04
		AG556	0.57 ± 0.23

[a] Immunoprecipitation.
[b] Relative activity of tyrosine kinase, as compared with untreated cells.

The results of these studies suggested that both the EGFR and Neu tyrosine kinase activities were present in anti-EGFR immunoprecipitates (Table I) and were consistent with previous findings in other cell types (16, 21), as well as studies in PC12 cells,[2] which indicated that EGF treatment induced the formation of EGFR-Neu heterodimers. This was further reinforced by the data shown in Table I, where the tyrosine kinase activity measured in anti-Neu immunoprecipitates was sensitive to both the EGFR and Neu antagonists. However, unlike the results obtained with EGFR and Neu immunoprecipitates from EGF-stimulated PC12 cells, the data obtained from HRG-stimulated cells were unexpected. In particular, immunoprecipitation of Neu from HRG-treated PC12 cells yielded tyrosine kinase activity that was not only sensitive to the Neu kinase inhibitor (AG879) but also to the EGFR kinase inhibitor (AG556) (Table I). This finding indicates the possibility that EGFR is present in anti-Neu immunoprecipitate, although HRG does not serve as a ligand for EGFR. Likewise, anti-EGFR immunoprecipitates from HRG-treated PC12 cells showed some sensitivity to the Neu kinase inhibitor (AG879) in addition to the EGFR kinase inhibitor (AG556) (Table I), indicating the presence of Neu in the immunoprecipitate.

Using insect cell expression systems for members of the EGFR family (EGFR, Neu, or ErbB4), we have verified that AG879 shows a strong preference for Neu *versus* EGFR and is incapable of inhibiting ErbB4 activity (Table II). However, both AG556 and AG1478, which were reported to be specific for the EGFR (31, 33), are also capable of inhibiting ErbB4 tyrosine kinase activity (Table II).

Thus, one possible explanation for the results obtained with the anti-Neu immunoprecipitates (*e.g.* Table I) was that HRG stimulated the formation of an ErbB4-Neu primary heterodimer, in which the tyrosine kinase activity of ErbB4 would be sensitive to AG556. Thus far, however, we have not been able to reliably detect the formation of a HRG-stimulated ErbB4-Neu heterodimer in PC12 cells through Western blot analysis, using specific anti-ErbB4 antibodies. An additional possibility, which would not be mutually exclusive with the first, was that HRG stimulated the formation of a Neu-EGFR heterodimer complex. In fact, as shown in Fig. 1 (also, see Fig. 2, below), anti-Neu immunoprecipitates from HRG-treated PC12 cells contain EGFR, as well as Neu and ErbB3.

[2] D. Gamett, unpublished data.

TABLE II

Effect of tyrphostins on tyrosine kinase activity of receptors expressed in insect cells

The values in the table represent relative tyrosine kinase activities, as compared with control systems without tyrphostin. Procedures for the determination of the enzymatic activities are described under "Materials and Methods" and in Refs. 28 and 29.

Tyrphostin	EGFR		Neu		ErbB4	
	S[a]	R[b]	S	R	S	R
AG556	0.23	0.11	1.21	1.03	0.32	0.35
AG1478	0.09	0.09	0.88	1.06	0.29	0.19
AG879	0.87	0.74	0.20	0.38	1.16	0.81

[a] Phosphorylation of a substrate (poly-Glu_4-Tyr_1).
[b] Receptor autophosphorylation.

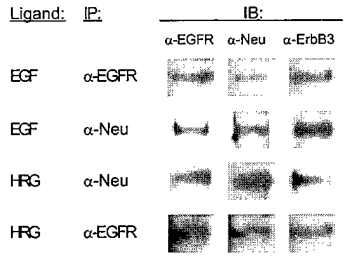

FIG. 1. **Receptors present in immunoprecipitates.** PC12 cells in culture were starved for 1 day in serum-free medium, detached, and suspended in serum-free medium. Either HRG (20 nM) or EGF (100 ng/ml) was added to the cells for 2 min, after which the cells were lysed, and the cell lysates were subjected to immunoprecipitation (*IP*) using either anti-EGFR or anti-Neu antibodies. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-EGFR, anti-Neu, or anti-ErbB3 antibodies followed by secondary horseradish peroxidase-conjugated antibodies. The bands were detected by chemiluminescence. Bands were not detected after immunoblotting with anti-ErbB4 antibodies.

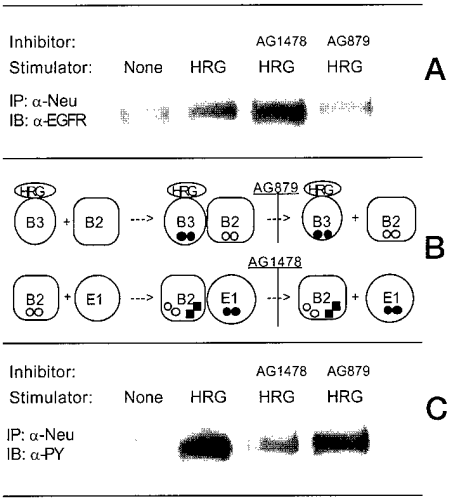

FIG. 2. **HRG induces the association of Neu with EGFR.** *A*, PC12 cells in culture were starved for 1 day in serum-free growth medium, detached, and suspended in serum-free medium. Some cell samples were preincubated for 2 min with tyrphostin AG1478 (0.5 μM) or AG879 (25 μM). Then the cells were stimulated for 2 min with HRG (20 nM) and lysed, and the cell lysates were subjected to immunoprecipitation (*IP*) using anti-Neu antibodies. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-EGFR antibodies, followed by secondary horseradish peroxidase-conjugated antibodies. The bands were detected by chemiluminescence. *B*, cell stimulation by HRG results in the formation of the primary heterodimer ErbB3-Neu, followed by trans-phosphorylation, dimer dissociation, and interaction of the phospho-activated Neu with EGFR to form the secondary heterodimer Neu-EGFR. The tyrphostin AG879 inhibits the tyrosine kinase activity of Neu and thus arrests the dissociation of the primary dimer Neu-ErbB3 and the formation of the secondary dimer Neu-EGFR. The tyrphostin AG1478 inhibits EGFR tyrosine kinase activity and thus arrests the dissociation of the secondary dimer Neu-EGFR. *E1*, EGFR; *B2*, Neu; *B3*, ErbB3. *Light gray* and *dark gray symbols* represent low and high phosphorylation levels, respectively. *C*, the immunoblot (*IB*) described in *A* was subjected to a second blotting with anti-phosphotyrosine antibodies.

Because neither the EGFR nor Neu directly binds HRG, these findings suggest that the EGFR-Neu complex is a secondary outcome of a primary heterodimerization event stimulated by HRG. We suggest that the trans-phosphorylation in the HRG-stimulated ErbB3-Neu dimer might be followed by dimer dissociation and interaction between activated Neu and a latent EGFR. This suggestion is in concert with our previous study, in which we have shown that HRG stimulates the heterodimerization between ErbB3 and Neu in PC12 cells but that following the trans-phosphorylation of ErbB3, it dissociated from Neu and formed a complex with the 85-kDa regulatory subunit (p85) of the phosphatidylinositol 3-kinase (26). Taken together, these results led us to hypothesize that the HRG-stimulated ErbB3-Neu (primary) heterodimer was not stable under conditions of trans-phosphorylation, such that the phosphorylation of the individual receptor (within the primary heterodimer) causes dimer dissociation, thus making the resulting monomers available to interact with other signaling partners. In some cases, the phosphorylated monomeric receptors may bind to Src homology 2 domain-containing proteins like p85. In other cases, however, the phosphorylated monomers can apparently interact with other (nonphosphorylated) receptor monomers to form secondary dimers.

If this hypothesis were correct, we would predict that treatment with different tyrphostins, specific inhibitors of tyrosine kinase activity (31), should have specific effects on the formation of secondary receptor dimers. The data presented in Fig. 2*A* (*lanes 3* and *4*) show that this in fact was the case. The treatment of PC12 cells with AG1478, which is a potent and specific antagonist for EGFR in various cells (31, 33, also see Table II), increased the formation of the HRG-stimulated Neu-EGFR secondary dimer, whereas, treatment with AG879, the Neu tyrosine kinase antagonist (31, 33, 34), strongly inhibited the formation of the Neu-EGFR secondary dimer. These findings are fully consistent with the model shown in Fig. 2*B*. Namely, the tyrphostin AG879, by inhibiting Neu tyrosine kinase activity, prevents both the autophosphorylation of Neu and the trans-phosphorylation of ErbB3 and thus prevents the dissociation of the primary Neu-ErbB3 dimer. This, then, inhibits the formation of secondary Neu-EGFR heterodimers. However, tyrphostin AG1478, by inhibiting the EGFR tyrosine kinase activity, prevents the trans-phosphorylation of Neu within the secondary Neu-EGFR dimer, hence inhibiting the dissociation of this dimer, and increases its amount.

Fig. 2*C* shows the results of a Western blot of Neu immunoprecipitates using an anti-phosphotyrosine antibody. *Lanes 1* and *2* show that HRG treatment of PC12 cells stimulated the tyrosine phosphorylation of the receptors present in the anti-Neu immunoprecipitate. This phosphorylation was strongly inhibited by AG1478 (Fig. 2*C*, *lane 3*), which argues that the majority of the phosphorylation occurred (in an EGFR-catalyzed manner) within the secondary Neu-EGFR dimer. The tyrosine phosphorylation of Neu was only weakly inhibited by

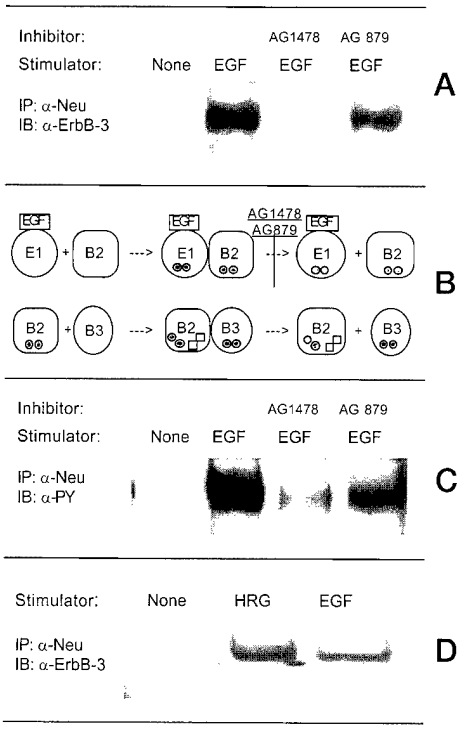

FIG. 3. **EGF induces the association of Neu with ErbB3.** *A*, PC12 cells in culture were starved for 1 day in serum-free growth medium, detached, and suspended in serum-free medium. The cells were stimulated for 2 min with EGF (100 ng/ml) and then lysed, and the cell lysates were subjected to immunoprecipitation (*IP*) using anti-Neu antibodies. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-ErbB3 antibodies, followed by secondary horseradish peroxidase-conjugated antibodies. The bands were detected by chemiluminescence. *B*, EGF induces the formation of the heterodimer Neu-EGFR. Trans-phosphorylation leads to dimer dissociation followed by the interaction of activated Neu with ErbB3 to form the secondary dimer Neu-ErbB3. The tyrphostins AG1478 and AG879 inhibit the trans-phosphorylation and the dissociation of the primary dimer EGFR-Neu and thus decrease the levels of the secondary dimer Neu-ErbB3. *E1*, EGFR; *B2*, Neu; *B3*, ErbB3. *Light gray* and *dark gray symbols* represent low and high phosphorylation levels, respectively. *C*, the immunoblot (*IB*) described in *A* was subjected to a second blotting with anti-phosphotyrosine antibodies. *D*, cells were stimulated for 2 min with either HRG or EGF (20 nM or 100 ng/ml, respectively). Then the cells were lysed, and the cell lysates were subjected to immunoprecipitation using anti-Neu antibodies. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-ErbB3 antibodies, followed by secondary horseradish peroxidase-conjugated antibodies. The bands were detected by chemiluminescence.

AG879 (Fig. 2*C*, *lane 4*), which suggests that Neu autophosphorylation represented only a small percentage of the total tyrosine phosphorylation of Neu.

The data shown in Fig. 3 provide evidence for the formation of another type of secondary dimer. In this case, the stimulation of PC12 cells with EGF led to the formation of a dimer between Neu and ErbB3. Again, because neither of these receptors bind EGF, this dimerization event must be the secondary outcome of a primary EGF-stimulated dimerization between the EGFR and Neu. The formation of this secondary receptor dimer was strongly inhibited by the EGFR antagonist, AG1478, and moderately inhibited by the Neu antagonist, AG879 (Fig. 3*A*). These results are consistent with the model depicted in Fig. 3*B*. The marked inhibition of (secondary) Neu-ErbB3 dimer formation by AG1478 suggests that the EGFR-catalyzed trans-phosphorylation of Neu is essential for the dissociation of Neu from the EGFR (within the primary dimer). The modest inhibition of (secondary) Neu-ErbB3 dimer formation by AG879 argues that Neu autophosphorylation and EGFR trans-phosphorylation (by Neu) are less critical for the

TABLE III
Selection of primary and secondary dimers by specific ligands and selective immunoprecipitation

Underlined receptors are present only in secondary dimers under the selective conditions used.

Ligand	IP[a]	Primary dimers	Secondary dimers
HRG	Anti-Neu	Neu-ErbB3	EGFR-Neu
		Neu-ErbB4	Neu-Neu
HRG	Anti-EGFR	EGFR-ErbB3	EGFR-EGFR
		EGFR-ErbB4	EGFR-Neu
EGF	Anti-Neu	EGFR-Neu	Neu-Neu
			Neu-ErbB3
			Neu-ErbB4
EGF	Anti-EGFR	EGFR-EGFR	
		EGFR-Neu	
		EGFR-ErbB3	
		EGFR-ErbB4	

[a] Immunoprecipitation.

dissociation of the primary EGFR-Neu dimer. The data presented in Fig. 3*C* are consistent with the notion that Neu is strongly (trans) phosphorylated by the EGFR within the primary EGFR-Neu heterodimer, as evidenced by almost complete elimination of tyrosine phosphorylation by AG1478. Neu autophosphorylation, however, apparently occurs to a lesser extent, because AG879 shows a modest effect. Studies performed with A431 membranes containing EGFR, and insect cell-expressed Neu have also shown that although the EGFR can strongly trans-phosphorylate Neu, there is little or no Neu-catalyzed trans-phosphorylation of the EGFR.[3]

The data presented in Fig. 3*D* compare the results of co-immunoprecipitation of ErbB3 with Neu from PC12 cells treated with HRG *versus* cells treated with EGF. The HRG stimulation would lead to the formation of an ErbB3-Neu (primary) heterodimer, and as previously reported, this yields a doublet in the ErbB3 Western blots that reflected different tyrosine phosphorylation states of ErbB3 (26). The lower mobility (*upper band*) is exclusively found with p85 (26), leading to the suggestion that this represents a phosphorylated form of ErbB3 that ultimately dissociates from Neu and forms a complex with p85. EGF induces the formation of a Neu-ErbB3 secondary dimer. In this case, the ErbB3 Western blot shows only a single band with a mobility essentially identical to the faster mobility ErbB3 band obtained in HRG-treated cells. Thus, these results demonstrate that the tyrosine phosphorylation of ErbB3 within Neu-ErbB3 heterodimers differs depending on whether it is a primary Neu-ErbB3 heterodimer or a secondary heterodimer, and presumably these differences will have important consequences regarding the specific substrates that are recruited to ErbB3.

There currently is little debate regarding the fundamental importance of growth factor-stimulated receptor dimer formation in the actions of members of the EGFR family or in the activities of a variety of other receptor tyrosine kinases. Recent studies have shown that receptor activation and dimerization are dependent on the ligand type (24) and that receptor dimerization is not a random process but is subjected to a certain hierarchy (16, 24). The data presented in this study now provide evidence that growth factors can actually stimulate a cascade of receptor dimerization events through a mechanism that reflects the enzyme-substrate nature of the interactions between members of the EGFR family. We propose that growth factor-stimulated receptor dimerization represents a transient rather than a stable interaction. The trans-phosphorylation of one receptor (*i.e.* the substrate) by its partner receptor (the enzyme) results in the dissociation of the receptor dimer (sim-

[3] P. Guy and K. Carraway, unpublished data.

ilar to the dissociation of an enzyme-product complex). This enables the individual, trans-phosphorylated receptor(s) to form new receptor dimers and to phosphorylate new partners. Table III shows that a variety of different primary and secondary receptor dimer combinations are possible, depending on the activating ligand used and the immunoprecipitating antibody. In the present work, we show that treatment of PC12 cells with HRG leads not only to the formation of an ErbB3-Neu primary receptor dimer but also to a Neu-EGFR secondary dimer and that treatment with EGF induces both a primary EGFR-Neu heterodimer and a secondary Neu-ErbB3 heterodimer.

The idea that primary receptor dimerization can give rise to secondary dimerization events is supported by various other lines of study. As alluded to above, data obtained from HRG-treated PC12 cells were consistent with the idea that the primary (HRG-stimulated) receptor dimer formed between ErbB3 and Neu was of a transient nature and upon dissociation leads to ErbB3-p85 interactions (26). A study of the reversible dimerization of the EGFR has shown that phosphorylated monomers appeared after receptor dimerization (19). This delayed appearance of phosphorylated EGFR monomers (as compared with the formation of phosphorylated dimers) could be attributed to the dissociation of the EGFR homodimer. Recent studies have also yielded data consistent with the idea that different members of the EGFR family can become phosphorylated and activated in a ligand-independent manner, *i.e.* in a manner somewhat analogous to the ligand-independent formation of secondary dimerization events between a phosphorylated/activated receptor monomer and a nonphosphorylated/inactive receptor monomer. Specifically, it has recently been shown that both the EGFR and Neu can be phosphorylated and activated (independent of EGF or HRG) by ligands for G protein-coupled receptors, presumably through a mechanism where a Src-like tyrosine kinase (activated downstream from the G protein-coupled receptor) binds and phosphorylates the EGFR or Neu (35). The secondary dimer formation is also in concert with the findings that Neu is subjected to trans-activation by both EGF and HRG receptors (36–38) as well as with the concept of lateral signaling within the cell membrane by combinatorial receptor interactions (16).

The ability to undergo both primary and secondary receptor dimerization events would provide a means by which each member of the EGFR family could form a heterogeneous population of phosphorylated receptor species (depending on the types of primary and secondary receptor dimers that are generated), thereby increasing the diversity of signaling pathways that can be initiated by a single growth factor. In addition, the signals induced by secondary receptor dimers will affect cells that have already been subjected to the first wave of signals emanating from the primary receptor dimerization events. Thus, it is conceivable that the second wave of signals might stimulate or arrest and elongate or terminate the effects induced by the first wave of signals. Thus, the mechanism of secondary dimerization provides an additional means for control and for fine tuning of signals initiated by the members of the EGFR family.

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Communication

The Pleckstrin Homology Domain Mediates Transformation by Oncogenic Dbl through Specific Intracellular Targeting*

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The pleckstrin homology (PH) domain is an ~100 amino acid structural motif found in many cellular signaling molecules, including the Dbl oncoprotein and related, putative guanine nucleotide exchange factors (GEFs). Here we have examined the role of the Dbl PH (dPH) domain in the activities of oncogenic Dbl. We report that the dPH domain is not involved in the interaction of Dbl with small GTP-binding proteins and is incapable of transforming NIH 3T3 fibroblasts. On the other hand, co-expression of the dPH domain with oncogenic Dbl inhibits Dbl-induced transformation. A deletion mutant of Dbl that lacks a significant portion of the PH domain retains full GEF activity, but is completely inactive in transformation assays. Replacement of the PH domain by the membrane-targeting sequence of Ras is not sufficient for the recovery of transforming activity. However, subcellular fractionations of Dbl and Dbl mutants revealed that the PH domain is necessary and sufficient for the association of Dbl with the Triton X-100-insoluble cytoskeletal components. Thus, our results suggest that the dPH domain mediates cellular transformation by targeting the Dbl protein to specific cytoskeletal locations to activate Rho-type small GTP-binding proteins.

The cytoskeletal-associated Dbl oncoprotein transforms NIH 3T3 cells (1) by activation of signaling pathways involving Rho-type GTP-binding proteins (2). Proto-Dbl is a 115-kDa cytoskeletal-associated protein that is found in brain, adrenal glands, and gonads (1). Oncogenic activation occurs as an outcome of an amino-terminal truncation of proto-Dbl, where a recombination event fuses about 10 kDa of an unidentified human gene product (from chromosome 3) on to the carboxyl-terminal half of Dbl (residues 498–925). The oncogenic Dbl protein contains at least two putative signaling motifs. The first is a region of 176 amino acids (residues 498–674) that was originally found to share significant homology with the *Saccharomyces cerevisiae* cell-division-cycle protein, Cdc24, and the breakpoint cluster region protein, Bcr[1] (3). This region, referred to as the Dbl homology (DH) domain, has been shown to be essential both for the transformation activity of oncogenic Dbl and for its ability to act as a GEF by stimulating the guanine nucleotide exchange activity of Cdc42Hs (4, 5). The second putative signaling motif is the pleckstrin homology (PH) domain (6, 7) and includes residues 703–812. Although PH domains appear to be relatively poorly conserved, both NMR and x-ray crystallographic studies of the PH domains of pleckstrin, dynamin, and spectrin indicate that they adopt a common three-dimensional structural motif (8–11).

Over the past few years, a growing family of oncogene products and other growth regulatory proteins have been shown to contain a DH domain in tandem with a PH domain. In addition to Cdc24 and Bcr, these include the Vav, Ost, Ect-2, Lbc, Lfc, and Dbs oncoproteins (12–17) and the activators of the Ras proteins, Sos (18), and Ras-GRF (19). All indications from previous studies are that the DH domain will form a binding site and in many cases contain GEF activity for Rho-like GTP-binding proteins (8, 9, 13, 14, 20–22). However, less is known about the roles of the PH domains. In the present study, we have used the Dbl oncoprotein as a model to examine the role of the PH domain in cellular transformation and GEF activity.

EXPERIMENTAL PROCEDURES

cDNA Transfection Studies—Transfection assays were done on duplicate cultures by adding 0.001, 0.01, 0.1, and 1 μg of DNA to the recipient NIH 3T3 cells using the Ca^{2+}-phosphate precipitation method (3). Foci (focus forming units) were scored 14 days after transfection, and the results were calculated as number of foci/pmol of DNA. The results listed in Fig. 1 and shown in Fig. 2*C* are the mean values of three transfection assays. Growth in soft agar was examined as described by Ron *et al.* (3).

Cellular Fractionation Studies—Control NIH 3T3 and different NIH 3T3 transfectants were lysed and fractionated into cytosolic (S), Triton X-100-solubilized membrane fractions (T), and Triton X-100-insoluble fractions (I) as described by Graziani *et al.* (1). Cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 3 h at 37 °C. Specific Dbl products were detected by immunoprecipitation using anti-Dbl-2 antibodies (3), electrophoresed through a 12% polyacrylamide gel and autoradiographed. For the detection of the PH domains (*e.g.* Figs. 2*B* and 4*D*, below), cells were immunoprecipitated with anti-Flag M5 antibodies and electrophoresed through a 15% polyacrylamide gel followed by immunoblotting with anti-Flag M5 antibodies.

Measurements of GDP Dissociation from Cdc42Hs—The [^{3}H]GDP dissociation assays were carried out as described previously (4, 5). In Fig. 3*A*, the amounts of GST-Dbl and GST-DH (see Fig. 1) purified from Sf9 insect cells were estimated by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis. ~200 nM of GST-Dbl or GST-DH were incubated with 1 μg of RhoA protein preloaded with [^{3}H]GDP in 100 μl of reaction buffer at room temperature, and 16-μl aliquots were diluted into 5 ml of ice-cold termination buffer (20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 100 mM NaCl) at various time points. In Fig. 3*B*, 1 μg

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[1] The abbreviations used are: Bcr, break point cluster region protein; DH, Dbl homology; PH, pleckstrin homology; GAPs, GTPase-activating proteins for low molecular mass GTP-binding proteins; GRF, guanine nucleotide-releasing factor; GEFs, guanine nucleotide-exchange factor for low molecular mass GTP-binding proteins; GST, glutathione *S*-transferase.

Appendix 2

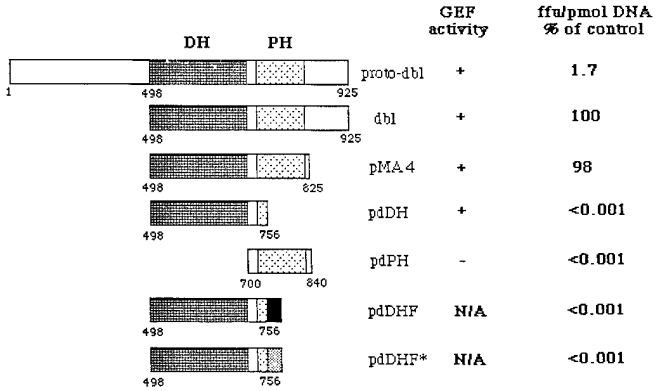

FIG. 1. **Schematic representations of oncogenic Dbl and different mutants of Dbl used in this study.** The abilities of these constructs to serve as GEFs for Rho and Cdc42Hs and to transform NIH 3T3 cells are summarized. pdDH represents the Dbl homology domain and pdPH is the pleckstrin homology domain. The abbreviation *ffu* represents focus forming units. 100% is 3×10^5 foci/pmol DNA. pDHF is a construct in which the PH domain sequences downstream from residue 750 are replaced with the carboxyl-terminal 16 amino acids of Ha-Ras, which include both the palmitylation and farnesylation sites (*black rectangle*). pdHF* is a construct encoding the DH domain of Dbl and the carboxyl-terminal 16 amino acids of Ha-Ras, except that the cysteine, which is normally farnesylated, has been changed to serine (*cross-hatched rectangle*). The GEF activity for these constructs has not been determined (*N/A*). The pdDH, pdDHF, and pdDHF* were subcloned from *dbl* by the polymerase chain reaction and inserted into the mammalian expression vector pZipneo. The GEF activities were measured as described (5) by the *in vitro* nitrocellulose filter binding assay either using the anti-Dbl immunoprecipitates from the NIH 3T3 transfectants (proto-Dbl, Dbl, and pMA4) or using the insect cell expressed peptides (DH and PH).

of [^{3}H]GDP-bound RhoA was incubated with 2 μM GST, 2 μM GST-PH (a fusion protein containing GST and the pleckstrin homology domain from the Dbl protein), 300 μM GST-Dbl, or 300 μM GST-Dbl and 2 μM GST-PH in a 100-μl reaction mixture.

RESULTS AND DISCUSSION

To investigate the role of the PH domain in cellular transformation mediated by the oncogenic Dbl protein, we analyzed several Dbl mutants for transforming activity in NIH 3T3 cells (Fig. 1). We found that while the transforming capability of a deletion mutant containing primarily the DH and PH domains (designated as *pMA4* in Fig. 1) was similar to that of the Dbl oncogene product, neither the DH domain nor the PH domain (*pdDH* and *pdPH*, respectively) alone showed any detectable effects on the growth of 3T3 fibroblasts. However, when Dbl and pdPH were co-expressed in NIH 3T3 cells (*dbl+pFlag/PHdbl*, Fig. 2*C*), we observed a significant reduction of the transforming activity by Dbl. Co-expression of Dbl with a Flag-tagged PH domain of the Dbl-related Vav oncoprotein (12), on the other hand, showed effects comparable with those obtained with the pFlag/neo vector control (Fig. 2*C*), even though it appeared to be more highly expressed (Fig. 2*B*, *lane 2*) than the PH domain of Dbl (Fig. 2*B*, *lane 1*). The level of expression of the Dbl oncoprotein was essentially equivalent in all cases (Fig. 2*A*).

To further confirm the selective inhibition of Dbl-induced transformation by the dPH domain, we used a second mammalian expression vector, pKH3 (23), to express the PH domain from either Dbl, Vav, or from the related yeast Cdc24 protein (20), together with the Dbl oncogene product. As shown in Fig. 2*C*, the expression of the dPH domain inhibited the focus-forming activity of oncogenic Dbl by ~40%, whereas co-expression of Dbl with the PH domain of Vav (pKH3/PHvav) had little effect. Mass populations of these transfected cells also were examined for their ability to display anchorage-independent growth. We observed that cells co-expressing Dbl and the dPH domain lost the ability to grow in soft agar (data not shown). In some cases, we found that expression of a Flag-tagged PH domain of Vav caused some inhibition of Dbl-induced growth in soft agar, suggesting that the Vav PH domain (perhaps when expressed at sufficient levels) was capable of competing with the PH domain of Dbl for a cellular target. However, it is likely that the Vav PH domain is a weak competitor, since we often observed no detectable effects with either the Flag-tagged protein or when expressing the PH domain of Vav from the pKH3 vector. We also have found no detectable effects on Dbl transformation when expressing the PH domain from Cdc24 (data not shown). Mass cultures of Dbl transfectants expressing the dPH domain also displayed a less transformed phenotype compared with Dbl transfectants alone or compared with cells co-expressing Dbl and the PH domain of Vav (data not shown). Taken together, these results suggest that the PH domain of Dbl behaves as a selective antagonist of Dbl-induced transformation, possibly by binding to a saturable and specific ligand in cells.

Previously we have shown that the DH domain alone is sufficient for the GEF activity for Cdc42Hs (5). Since oncogenic Dbl also stimulates the guanine nucleotide exchange activity of Rho, we examined whether the Dbl domain is sufficient for stimulating the activation of Rho. To do this, we compared Rho-GEF activities of approximately equal amounts (~200 nM) of insect cell-expressed, purified GST-Dbl and GST-DH domain. No significant differences were observed for the abilities of the GST-Dbl and GST-DH to stimulate [^{3}H]GDP dissociation from RhoA (Fig. 3*A*). These results suggest that the PH domain does not contribute to the GEF function of Dbl. This is further reinforced by the results in Fig. 3*B*, which show that the addition of excess *Escherichia coli* recombinant PH domain to GEF assay mixtures containing [^{3}H]GDP-bound RhoA and recombinant GST-Dbl has no detectable effect on the time-course of GST-Dbl-stimulated [^{3}H]GDP dissociation from RhoA. The GST-PH domain, alone, also shows no ability to stimulate [^{3}H]GDP dissociation from RhoA (compared with GST alone). Similar results were also obtained with [^{3}H]GDP-bound Cdc42Hs (data not shown). Thus, the dPH domain is not involved in the interactions of Dbl with RhoA and Cdc42Hs or in the direct regulation of the GEF catalytic activity of the DH domain.

The membrane association of βARK and spectrin has been attributed to their PH domains (24, 25). The PH domains of βARK, BTK, PLCγ, IRS-1, and Ras-GRF have been shown to bind to plasma membrane-associated $\beta\gamma$ subunits of the heterotrimeric G-proteins (26, 27), and they all behave as antagonists of G$\beta\gamma$-mediated signaling (28). Recent evidence also suggests that PH domains from many signaling molecules including βARK and Ras-GAP can bind to specific phospholipids, namely phosphatidylinositol 4,5-bisphosphate (PIP_2) (29). These findings raised the possibility that the PH domain mediates the membrane targeting of oncogenic Dbl. It has been shown that the introduction of a membrane-targeting sequence into the Ras GEFs, Cdc25 and Sos (30, 31), was sufficient to activate Ras, and more recently, that the addition of a membrane-targeting sequence in place of the PH domain of the Lfc oncoprotein was able to restore full transformation activity (32). Thus, we examined whether the substitution of the dPH domain with a membrane-targeting sequence enabled the DH domain of Dbl to induce transformation. A chimeric molecule containing the DH domain (residues 498–756) fused to the Ras membrane-targeting farnesylation signal sequence (designated *pdHF* in Fig. 1) was constructed and assayed for focus-forming activity in NIH 3T3 cells. This chimera was expressed at a comparable level to oncogenic Dbl and a percentage (10–20%) of the total chimeric molecules was targeted to the membrane

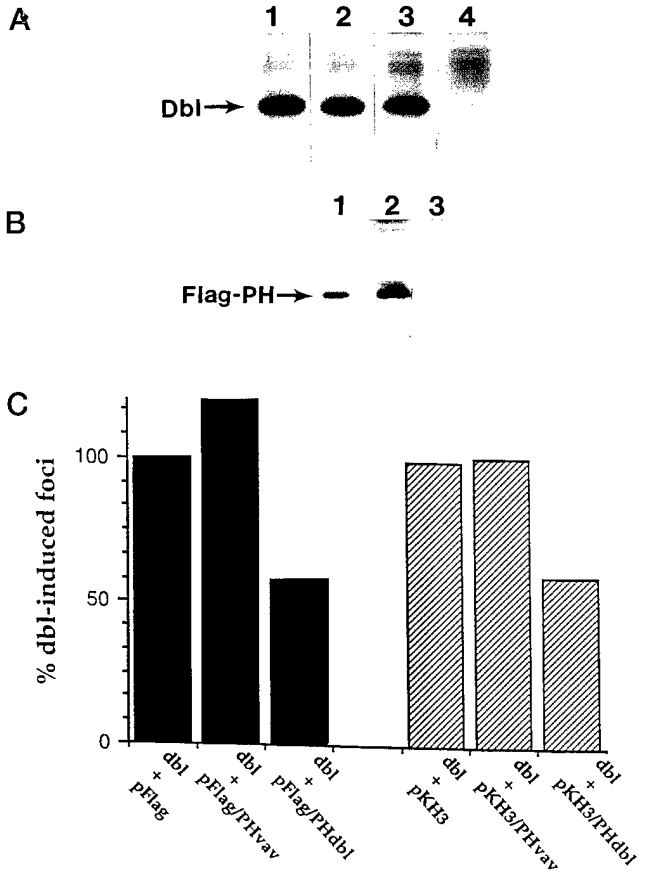

FIG. 2. **Expression of the PH domain inhibits Dbl-induced transformation in NIH 3T3 cells.** *A*, detection of the Dbl oncoprotein (using an anti-Dbl antibody) in NIH 3T3 transfectants. *Lane 1* represents cells expressing the Dbl oncoprotein. *Lane 2* represents cells co-expressing Dbl and the Dbl PH domain (dPH). *Lane 3* represents cells co-expressing Dbl and the Vav PH domain. *Lane 4* is a control, *i.e.* cells transfected with the plasmid (pFlag) used to express the PH domains. *B*, detection of the PH domains of Dbl and Vav (using M5 anti-Flag antibody) in NIH 3T3 cells. *Lane 1* represents cells co-expressing Dbl and the Dbl PH domain (dPH). *Lane 2* represents cells co-expressing Dbl and the Vav PH domain. *Lane 3* is a control (cells transfected with the pFlag plasmid). *C*, effects of the PH domains of Dbl and Vav on Dbl-induced foci-formation. pFlag/PHdbl and pKH3/PHdbl represent the mammalian expression vectors encoding the Dbl PH domain and pFlag/PHvav and pKH3/PHvav are the expression vectors encoding the Vav PH domain. The results shown represent the average of three independent experiments.

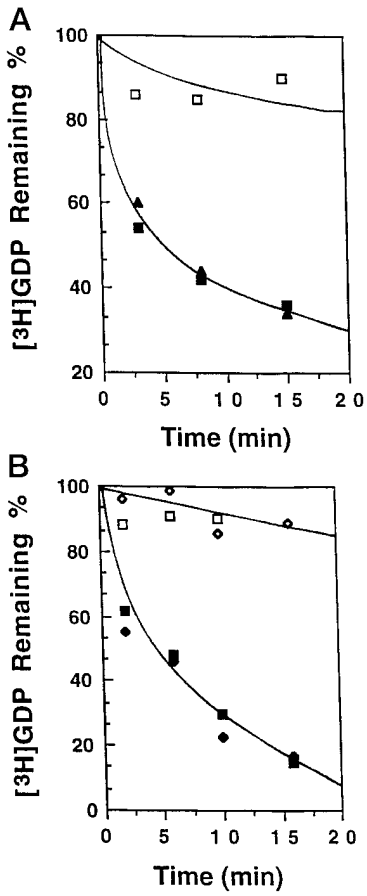

FIG. 3. **The PH domain is not directly involved in the regulation of the GEF activity of the Dbl oncoprotein.** *A*, comparison of the abilities of oncogenic Dbl (■) and the DH domain of Dbl (▲) to stimulate [^{3}H]GDP dissociation of RhoA. The dissociation of [^{3}H]GDP from RhoA, alone, is depicted by (□). *B*, effect of the PH domain on the kinetics of Dbl-stimulated [^{3}H]GDP dissociation from RhoA. ■ represents Dbl-stimulated GDP dissociation in the absence of the PH domain and ♦ represents Dbl-stimulated GDP dissociation in the presence of the PH domain. □ and ◇ represent the corresponding controls for RhoA in the absence of Dbl.

surface (*i.e.* the Triton X-100 solubilized fraction (*T*) in Fig. 4*A*). However, this did not restore transforming activity to the DH domain (Fig. 1). Although, one possible explanation is that the amount of the chimera expressed at the membrane surface was not sufficient to stimulate a transforming signal, this does not seem likely based on what we have observed regarding the range of expression of oncogenic Dbl that will yield cellular transformation (34).

We have reported previously that significant portions of both proto- and oncogenic Dbl are localized to the Triton X-100-insoluble fractions of transfected NIH 3T3 cells, suggesting an association with the cytoskeletal matrix (1). To address the possible role of the dPH domain in mediating this pattern of localization for the Dbl protein, stable transfectants of Dbl and Dbl deletion mutants (Fig. 1) were subjected to subcellular fractionation. The crude membrane fractions (P100) of the cells were solubilized either by 1% Triton X-100 or by treatment with 0.1% SDS and 0.25% sodium deoxycholate. Anti-Dbl immunoprecipitates revealed that a percentage of both the intact oncogenic Dbl protein and a truncation mutant pMA4 associated with the Triton X-100-insoluble fractions of cells (designated by *I* in Fig. 4, *B* and *C*). The amounts of oncogenic Dbl and pMA4 that were present in the Triton X-100-insoluble fraction typically varied between 50 and 70% of the total detectable protein, although in some cases (Fig. 4*B*) the percentage of oncogenic Dbl in this fraction was less than 50%. However, the DH domain of Dbl, which lacks transforming ability, was localized exclusively to the cytosolic fraction (designated *S* in Fig. 4*C*). When cells expressing the Flag-tagged PH domains were subjected to similar fractionation, the PH domains were found associated with the Triton X-100-insoluble fractions (Fig. 4*D*). These results suggest that the dPH domain is directly responsible for the association of oncogenic Dbl with the Triton X-100-insoluble cytoskeletal fraction and thus may serve to target the catalytic DH domain to the cytoskeleton.

We have reported previously that the DH domain is responsible for Dbl GEF function and is required for Dbl transforming activity (3, 5). Here, we demonstrate that while the dPH domain does not seem to be involved in the interactions of Rho-type small GTP-binding proteins with Dbl, it is essential for Dbl transforming activity. Thus, our present findings establish that both the DH and PH domains are required for the cellular function of Dbl. Indeed, the minimum structural unit (pMA4) of oncogenic Dbl conferring complete transforming activity just

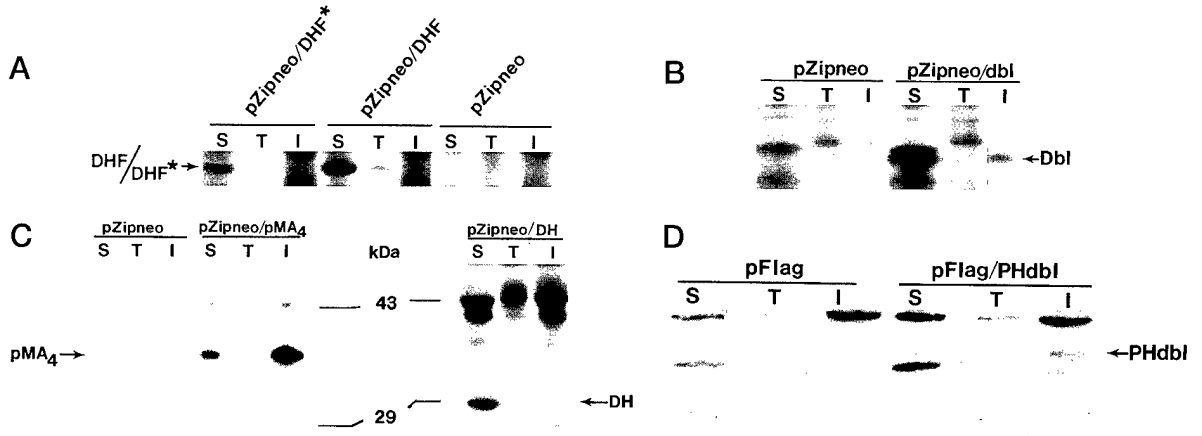

FIG. 4. **The PH domain mediates the cytoskeletal association of the Dbl oncoprotein.** *A*, the membrane attachment signal from Ha-Ras targets the DH domain to the Triton X-100-soluble fraction from cell membranes. pZIPneo/DHF (see Fig. 1) represents the construct encoding the DH domain of Dbl and the carboxyl-terminal 16 amino acids of Ha-Ras, including the palmitylation and farnesylation sites. pZIPneo/DHF* represents the construct encoding the DH domain of Dbl and the carboxyl-terminal 16 amino acids of Ha-Ras (except that the cysteine which serves as the farnesylation site has been changed to serine). *S* represents the soluble fraction, *T* is the Triton X-100-soluble fraction from membranes, and *I* is the Triton X-100-insoluble fraction. *B*, oncogenic Dbl is associated with the Triton X-100-insoluble fraction of cells. *C*, fractionations of the pMA4 and DH domain transfectants. *D*, fractionation of cells expressing the Dbl PH domain. The data shown in *A–C* were obtained by immunoprecipitating the Dbl proteins with the anti-Dbl antibody from cells that were labeled with [^{35}S]methionine and [^{35}S]cysteine. The data shown in *D* represent immunoblots using the anti-Flag M5 antibody.

encompasses the DH domain and the PH domain. The finding that plasma membrane-targeting of Dbl is not sufficient to confer transforming activity, coupled with the requirement of the dPH domain as the necessary and sufficient element for association of the Dbl protein with the Triton X-100-insoluble component, suggests that the function of the PH domain resides in its ability to target the catalytic DH domain to the cytoskeletal matrix. Whether this targeting function holds for other members of Dbl-related GEF family proteins remains to be seen. However, based on the observation that the PH domains of Dbl-related molecules Vav and Cdc24 do not act effectively as inhibitors of Dbl-induced transformation, it is an attractive possibility that different members of the family of Dbl-related proteins may be targeted by their PH domains to distinct cellular locations to activate various Rho-type GTP-binding proteins, in response to different extracellular stimuli such as epidermal growth factor, platelet-derived growth factor, lysophosphatidic acid, and bradykinin. This may also explain the finding that substitution of a membrane-targeting (*i.e.* Ras-farnesylation) sequence for the PH domain of Lfc restored its transformation capability (32), whereas this substitution did not restore transforming activity to a Dbl protein that just contains the DH domain. It may be that Lfc needs to be targeted to the plasma membrane to optimally couple to other protein components in its signaling pathway while Dbl needs to be targeted to a cytoskeletal location.

The identity of the ligand(s) that binds to the PH domain of oncogenic Dbl will represent an important focus of future studies. It seems likely, that given the hypervariable nature of the putative ligand-binding cleft in the PH domains that have thus far been identified (33), a complex diversity of ligands may exist that are responsible for mediating the actions of various PH domain-containing signaling molecules, including Dbl and related regulatory molecules of small GTP-binding proteins.

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Phosphatidylinositol 4,5-bisphosphate Provides an Alternative to Guanine Nucleotide Exchange Factors by Stimulating the Dissociation of GDP from Cdc42Hs*

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Members of the Rho subfamily of Ras-related GTP-binding proteins play important roles in the organization of the actin cytoskeleton and in the regulation of cell growth. We have shown previously that the *dbl* oncogene product, which represents a prototype for a family of growth regulatory proteins, activates Rho subfamily GTP-binding proteins by catalyzing the dissociation of GDP from their nucleotide binding site. In the present study, we demonstrate that the acidic phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), provides an alternative mechanism for the activation of Cdc42Hs. Among a variety of lipids tested, only PIP_2 was able to stimulate GDP release from Cdc42Hs in a dose-dependent manner, with a half-maximum effect at ~50 μM. Unlike the Dbl oncoprotein, which requires the presence of (free) guanine nucleotide in the medium to replace the GDP bound to Cdc42Hs, PIP_2 stimulates GDP release from Cdc42Hs in the absence of free guanine nucleotide. PIP_2, when incorporated into phosphatidylcholine carrier vesicles, binds tightly to the guanine nucleotide-depleted form of Cdc42Hs and weakly to the GDP-bound form of the GTP-binding protein but does not bind to GTP-bound Cdc42Hs, similar to what was observed for the Dbl oncoprotein. However, mutational analysis of Cdc42Hs indicates that the site that is essential for the functional interaction between PIP_2 and Cdc42Hs is distinct from the Dbl-binding site and is located at the positively charged carboxyl-terminal end of the GTP-binding protein. The GDP-releasing activity of PIP_2 is highly effective toward Cdc42Hs and Rho (and is similar to the reported effects of PIP_2 on Arf (Terui, T., Kahn, R. A., and Randazzo, P. A., (1994) *J. Biol. Chem.* 269, 28130–28135)), is less effective with Rac, and is not observed with Ras, Rap1a, or Ran. The ability of PIP_2 to activate Cdc42Hs (or Rho) and Arf provides a possible point of convergence for the biological pathways regulated by these different GTP-binding proteins and may be related to the synergism observed between Arf and Rho-subtype proteins in the stimulation of phospholipase D activity (Singer, W. D., Brown, H. A., Bokoch, G. M., and Sternweis, P. C. (1995) *J. Biol. Chem.* 270, 14944–14950).

The Rho subfamily of Ras-related GTP-binding proteins, which includes RhoA, Rac1, and Cdc42Hs, has been shown to regulate a diversity of cellular functions ranging from actin-mediated cytoskeletal rearrangements (Hall, 1994) to the stimulation of nuclear mitogen-activated protein kinases (Coso *et al.*, 1995; Minden *et al.*, 1995; Bagrodia *et al.*, 1995; Zhang *et al.*, 1995), transcription (Hill *et al.*, 1995), and DNA synthesis (Olson *et al.*, 1995). It is now well established that Rac1 is essential for growth factor-stimulated membrane ruffling and lamellipodia formation (Ridley *et al.*, 1992) and acts downstream from Ras in the stimulation of cell growth (Qui *et al.*, 1995), whereas RhoA controls the formation of stress fibers and focal adhesion complexes (Ridley and Hall, 1992). Cdc42 has been shown to be essential for bud-site assembly in *Saccharomyces cerevisiae* (Johnson and Pringle, 1990), for unidirectional and bidirectional cell growth in *Schizosaccharomyces pombe* (Miller and Johnson, 1994), and for filopodia formation in mammalian cells (Nobes and Hall, 1995; Kozma *et al.*, 1995). The macromolecular targets for the Rho subfamily GTP-binding proteins are just now beginning to be identified. Both GTP-bound Rac1 and Cdc42Hs bind to the M_r 85,000 regulatory subunit (p85) of the phosphatidylinositol 3-kinase (Zheng *et al.*, 1994a; Tolias *et al.*, 1995), to the p70 S6 kinase (Chou and Blenis, 1996), and to members of the p21-activated serine/threonine kinase (PAK) family (Manser *et al.*, 1994; Martin *et al.*, 1995; Bagrodia *et al.*, 1995). The GTP-bound form of RhoA also has been reported to stimulate phosphatidylinositol 3-kinase activity in platelets (Zhang *et al.*, 1993) as well as phosphatidylinositol 4-phosphate 5-kinase activity (Chong *et al.*, 1994). In addition, it recently has been shown that RhoA and Cdc42Hs can act synergistically with Arf to stimulate phospholipase D activity (Singer *et al.*, 1995).

Understanding how these different target activities are stimulated and then integrated to yield cytoskeletal changes and nuclear activities represents a formidable challenge. One approach is to determine how Rho subfamily GTP-binding proteins are activated, since this should represent the first key step in stimulating their target activities. One mode of activation of Rho-subtype GTP-binding proteins occurs through the stimulation of GDP dissociation (and consequently GTP/GDP exchange) by the family of Dbl-related proteins. The prototype for this family of guanine nucleotide exchange factors (GEFs)[1] is the Dbl oncoprotein, which was shown to act as a GEF for Cdc42Hs and RhoA (Hart *et al.*, 1991; Hart *et al.*, 1994). At present, 15 members of the Dbl-related family have been identified and characterized (Cerione and Zheng, 1996), with each member containing the characteristic Dbl homology domain in tandem with a Pleckstrin homology domain. Many of these

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[1] The abbreviations used are: GEF, nucleotide exchange factor; PIP_2, phosphatidylinositol 4,5-bisphosphate; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-*O*-(thiotriphosphate); PC, phosphatidylcholine.

proteins have been shown to have GEF activity including Cdc24 (Zheng *et al.*, 1994b), Ost (Horii *et al.*, 1994), Tiam-1 (Michiels *et al.*, 1995), and Lbc (Zheng *et al.*, 1995). Similar to the case for the interactions between heterotrimeric GTP-binding proteins (G proteins) and agonist-stimulated heptahelical receptors (Gilman, 1987), the Dbl-related GEFs bind preferentially to and stabilize the guanine nucleotide-depleted states of Rho-like GTP-binding proteins.

It is interesting that many of these Dbl-related proteins show a selective tissue distribution, whereas many of the Rho-subtype proteins (*e.g.* Cdc42Hs, RhoA, and Rac1) appear to be ubiquitous. This suggests that additional but as yet undiscovered Dbl-related proteins exist and/or that other mechanisms may be used to stimulate the activation of Rho-subtype GTP-binding proteins. In the present study, we describe one such potential alternative mechanism where the lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) is able to strongly stimulate GDP dissociation from Cdc42Hs and RhoA.

EXPERIMENTAL PROCEDURES

Materials—The small GTP-binding proteins Cdc42Hs, RhoA, Rac1, and Ha-Ras were expressed and purified as glutathione *S*-transferase (GST) fusion proteins from *Escherichia coli* as described previously (Hart *et al.*, 1994). The cDNAs encoding the K-Ras protein and Ras-GRF were kind gifts from Dr. Larry Feig (Tufts Medical School) and were expressed in *E. coli* as GST fusion proteins. The Rap1a protein was obtained from Dr. P. Polakis (Onyx Pharmaceutical, Emoryville, CA). The cDNA encoding Ran was the generous gift of Dr. M. Rush (New York University Medical Center, New York, NY). To express Ran in *E. coli* as a GST fusion protein, restriction sites for *Nco*I and *Hin*dIII were introduced immediately adjacent to the initiation and termination codons, respectively, using the polymerase chain reaction. The polymerase chain reaction product (730 base pairs) was then ligated with *Nco*I/*Hin*dIII-digested pGEX-KG (Pharmacia Biotech Inc.), and the resultant GST-Ran fusion protein was purified from transformed *E. coli* (JM101). Automated DNA sequencing revealed no mutations in the polymerase chain reaction product. The anti-Cdc42Hs polyclonal antibodies were raised against the unique carboxyl-terminal sequences of Cdc42Hs as described (Shinjo *et al.*, 1990). GST-Dbl protein was expressed in a baculovirus/Sf9 insect cell system (Hart *et al.*, 1994), and the Cdc42Hs-GTPase-activating protein was expressed and purified from *E. coli* (Barfod *et al.*, 1993). The ΔC7 Cdc42Hs truncation mutant was generated by polymerase chain reaction using the plaque-forming unit DNA polymerase (Stratagene), and the resulting sequences were verified through fluorescence automated sequencing. Lipids were purchased either from Sigma or from Avanti Polar Lipids. All lipids were dissolved in chloroform, dried under nitrogen, and resuspended by sonication in 50 mM Tris-HCl (pH 8.0) immediately prior to use. The pH was adjusted as necessary with NaOH. Radioisotope-labeled guanine nucleotides were obtained from DuPont NEN.

GDP Dissociation and GTP/GDP Exchange Assays—GDP dissociation and GTP/GDP exchange assays were carried out as described previously (Hart *et al.*, 1991). Two μg of Cdc42Hs loaded with [^{3}H]GDP was incubated with buffer mixtures containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 5 mM $MgCl_2$ (buffer A) with various lipids or Dbl proteins for the indicated times at room temperature. Assays monitoring the dissociation of GDP were stopped by dilution (20-μl aliquots) into 10-ml ice-cold buffer A, and the protein-bound nucleotide was trapped by filtration on nitrocellulose filters. For GTP/GDP exchange assays, 1 mM GTP was also included in the reaction buffer.

GTPγS Binding Assays—Assays monitoring the dissociation of GTPγS from Cdc42Hs were performed as described above for GDP, except that [^{35}S]GTPγS was used, and the concentration of Cdc42Hs-GTPγS was ~0.4 μM. GTPγS binding was determined as in Hart *et al.* (1991). Two μg of GDP-bound Cdc42Hs were incubated in buffers containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 10 μM [^{35}S]GTPγS (~5000 cpm/pmol), and 100 μM PIP_2, 0.5 μM GST-Dbl, or 100 μM phosphatidylcholine (PC) at 24 °C, and binding was determined at various time points.

Liposome·Protein Complex Formation Assays—Direct binding of liposome vesicles containing PIP_2 to Cdc42Hs was carried out by adapting the centrifugation protocol by Harlan *et al.* (1994). Briefly, Cdc42Hs was first loaded with GDP or GTPγS or depleted with nucleotide (Hart *et al.*, 1994). One μg of Cdc42Hs was then added to lipid vesicles (100 μl of total volume) with 500 μM carrier PC alone or 100 μM PIP_2 incorporated into the PC carrier vesicles through co-sonication. The mixture was incubated for 5 min before centrifugation in an ultraspeed Beckman airfuge (100,000 × *g*) for 30 min. The vesicles pelleted with this treatment were subjected to SDS-polyacylamide gel electrophoresis and anti-Cdc42Hs Western blot analysis, and the blot was visualized by the ECL method (DuPont NEN).

RESULTS AND DISCUSSION

The mode of regulation of Rho family GTP-binding proteins has been an area of intense research investigation (Boguski and McCormick, 1993) due to the involvement of these GTP-binding proteins in the stimulation of cytoskeletal changes and transcriptional activities as well as in the regulation of cell growth. In addition to protein factors that directly stimulate the guanine nucleotide exchange activities of Rho-subtype GTP-binding proteins, for which the Dbl oncoprotein is a prototype (Cerione and Zheng, 1996), various phospholipids have been implicated in the regulation of the Rac GTP-binding proteins through their effects on the GDP dissociation inhibitor molecule (Chuang *et al.*, 1993). In addition, the GTP-binding protein Arf, which undergoes GDP dissociation in response to PIP_2, appears to act synergistically with Rho-subtype proteins to stimulate (in a PIP_2-sensitive manner) phospholipase D activity (Malcolm *et al.*, 1994; Singer *et al.*, 1995; Moss and Vaughan, 1995). The latter finding is particularly interesting since we recently have found that Cdc42Hs is predominantly localized to Golgi membranes in mammalian cells and that its localization is influenced by different Arf mutants in a manner suggesting some type of interplay between Arf and Cdc42Hs.[2] Given these findings, we examined whether various phospholipids had any effect on the GTP-binding/GTPase cycle of Cdc42Hs.

Fig. 1*A* shows the results obtained when examining the effects of a panel of phospholipids on the rate of [^{3}H]GDP dissociation from Cdc42Hs. Only PIP_2 showed a significant stimulation of GDP dissociation from Cdc42Hs. Essentially no effect was observed when phosphatidic acid, phosphatidylethanolamine, phosphatidylserine, or phosphatidylinositol were incubated with [^{3}H]GDP-bound Cdc42Hs. We also have not detected any effects with inositol trisphosphate under conditions where PIP_2 strongly stimulates [^{3}H]GDP dissociation; however, PIP, at concentrations >500 μM, caused a slight increase in the rate of [^{3}H]GDP dissociation (data not shown).

Fig. 1*B* shows that the PIP_2-stimulated dissociation of GDP from Cdc42Hs is dose-dependent, with a half-maximal effect occurring at ~50 μM PIP_2. At PIP_2 levels ≥200 μM, the rate of GDP release from Cdc42Hs was increased ~10 fold.

The stimulation of GDP dissociation by PIP_2 was similar to that elicited by the oncogenic Dbl protein (Hart *et al.*, 1994). Fig. 2*A* shows time courses for the dissociation of [^{3}H]GDP from Cdc42Hs in the absence of activators or in the presence of the Dbl oncoprotein (~0.5 μM) or PIP_2 (100 μM). In these experiments, the dissociation of GDP was assayed in the presence of 1 mM GTP in the medium. Under these conditions, the half-time for [^{3}H]GDP dissociation from Cdc42Hs (in the presence of phosphatidylcholine as a control) was ~25 min, whereas Dbl-catalyzed GDP dissociation occurred with a half-time of ~1.5 min, and PIP_2-stimulated GDP dissociation occurred with a half-time of ~2.5 min.

It is interesting that when GDP dissociation was assayed in the absence of medium GTP, dramatic differences were observed between Dbl and PIP_2 (Fig. 2*B*). Specifically, PIP_2 was still capable of providing a strong stimulation of the initial rate of [^{3}H]GDP dissociation, whereas oncogenic Dbl failed to induce any detectable stimulatory effect.

These findings can be considered within the context of the

[2] Erickson, J. W., Zhang, C.-J., Kahn, R. A., Evans, T., and Cerione, R. A. (1996) *J. Biol. Chem.* **271,** in press.

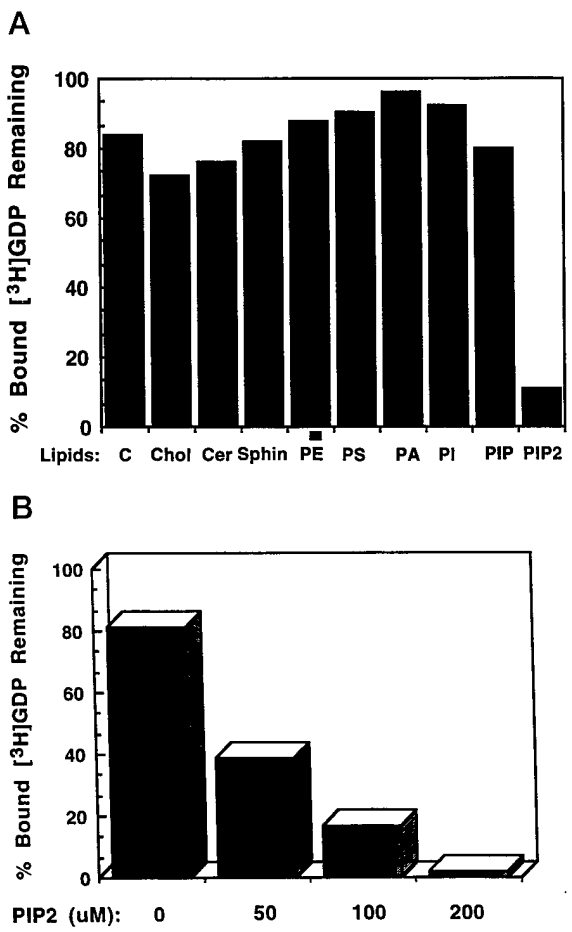

FIG. 1. **Effects of lipids on guanine nucleotide dissociation from Cdc42Hs.** In *A*, purified lipids at a concentration of 200 μM in buffer containing 20 mM Tris, pH 8.0, 100 mM NaCl, and 5 mM $MgCl_2$ were added to the incubation mixture with 2 μg *E. coli*-expressed [^{3}H]GDP-Cdc42Hs at a final concentration of 100 μM, and the reactions were terminated after 8 min at room temperature. *B*, dose-dependent [^{3}H]GDP dissociation from Cdc42Hs stimulated by PIP_2. GDP dissociation reactions were terminated 10 min after mixing PIP_2 and [^{3}H]GDP-bound Cdc42Hs.

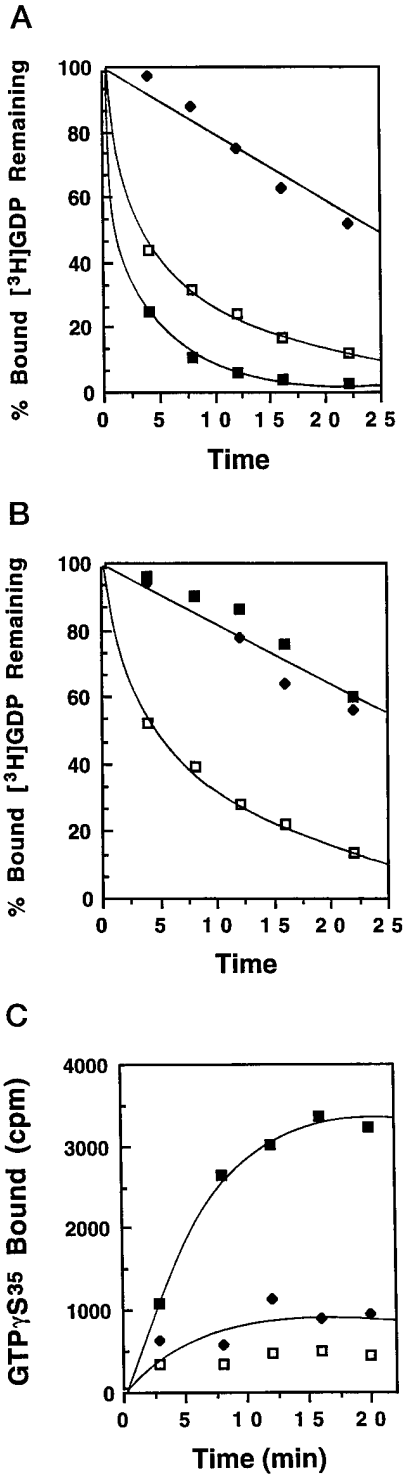

FIG. 2. **Comparisons of the guanine nucleotide exchange activities of Cdc42Hs stimulated by PIP_2 or the Dbl oncoprotein.** *A*, time courses of [^{3}H]GDP release from 2 μg Cdc42Hs stimulated by 100 μM PIP_2 (□), 0.5 μM GST-Dbl (■), or 200 μM PC (◆) under the GTP/GDP exchange assay conditions (with 1 mM free GTP). *B*, time courses of [^{3}H]GDP release from 2 μg Cdc42Hs stimulated by 100 μM PIP_2 (□), 0.5 μM GST-Dbl (■), or 200 μM PC (◆) in the absence of free nucleotides. *C*, effects of PIP_2 and Dbl on [^{35}S]GTPγS binding to Cdc42Hs. Cdc42Hs was preloaded with GDP, and the time courses for the binding of [^{35}S]GTPγS to Cdc42Hs in the presence of 100 μM PIP_2 (□), 0.5 μM GST-Dbl (■), or 200 μM PC (◆) were determined.

generally accepted model for the interactions of guanine nucleotides and GEFs with GTP-binding proteins. It typically has been assumed that GEFs act as antagonists of guanine nucleotide binding and that conversely, nucleotide binding weakens the interactions of GEFs with GTP-binding proteins. Thus, although Dbl weakens the affinity of GDP for Cdc42Hs, the amount of GDP present (*i.e.* initially bound to Cdc42Hs) is sufficient to maintain occupancy of the nucleotide site, even in the presence of this GEF. However, when a high excess of GTP is included in the medium, it effectively competes with GDP for the nucleotide site, thereby resulting in Dbl-catalyzed GTP-GDP exchange. This differs from the case for PIP_2, where this putative GEF is apparently more effective than Dbl in weakening the affinity of Cdc42Hs for guanine nucleotides. PIP_2 strongly stabilizes the guanine nucleotide-depleted state of Cdc42Hs; thus, it is difficult to observe a PIP_2-stimulated exchange of GDP for [^{35}S]GTPγS. In fact, under conditions where Dbl is able to elicit a strong stimulation of [^{35}S]GTPγS binding (when [GTPγS] = 0.5–1 μM), the addition of PIP_2 shows no detectable stimulation of GTPγS binding (Fig. 2*C*). In addition, although PIP_2 is more effective than Dbl in weakening the binding of guanine nucleotides to Cdc42Hs, it appears that guanine nucleotides also weaken the binding of PIP_2 to a greater extent than the binding of Dbl. Thus, although Dbl shows a weak but measurable stimulation of [^{35}S]GTPγS dissociation from Cdc42Hs, the addition of PIP_2 (at 50–100 μM) has no effect (data not shown).

Taken together, the results presented in Fig. 2 would suggest

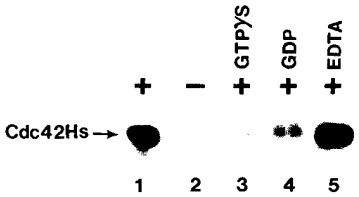

FIG. 3. **PIP_2 binds to the guanine nucleotide-depleted form of Cdc42Hs.** One μg of nucleotide-depleted Cdc42Hs (*EDTA lane*), Cdc42Hs bound to GDP (*GDP lane*), or Cdc42Hs bound to GTPγS (*GTPγS lane*) was incubated with 100 μM PIP_2 incorporated in PC vesicles for 10 min before ultracentrifugation in an airfuge. The resulting pellets were subjected to an anti-Cdc42Hs Western blot. *Lane −*, Cdc42Hs depleted of nucleotide incubated with PC vesicles alone; *lane +*, 0.1 μg Cdc42Hs as a positive control.

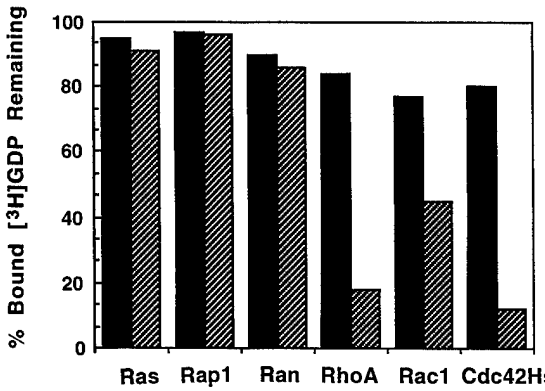

FIG. 4. **PIP_2 stimulates GDP dissociation from the Rho family GTP-binding proteins, Cdc42Hs and RhoA.** One μg of purified Ha-Ras, Rap1a, Ran, RhoA, Rac1, or Cdc42Hs were incubated with 100 μM PIP_2 under the GDP dissociation assay conditions for 8 min.

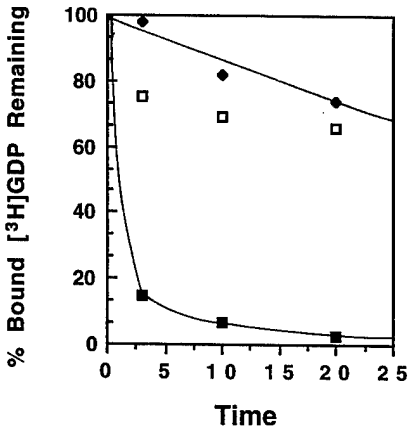

FIG. 5. **The carboxyl-terminal domain of Cdc42Hs is necessary for its interaction with PIP_2.** One μg of a carboxyl-terminal truncated (CΔ7) mutant of Cdc42Hs preloaded with [^{3}H]GDP was incubated with 100 μM PIP_2 (□), 0.5 μM GST-Dbl (■), or 1 μM GST (◆), and aliquots of the reaction mixtures were added to the termination buffers at the indicated time points.

that PIP_2 is capable of a direct interaction with Cdc42Hs and that this interaction is significantly influenced by the guanine nucleotide bound to the GTP-binding protein. Fig. 3 shows the results of such a direct binding experiment, using PC vesicles containing PIP_2. In this experiment, PC vesicles alone and PC vesicles containing PIP_2 were first incubated with Cdc42Hs, either in the guanine nucleotide-depleted state or in the GDP-bound state or the GTPγS-bound state, for 10 min at room temperature before pelleting the lipid vesicles by ultracentrifugation. Western blotting the pelleted vesicles with a specific anti-Cdc42Hs antibody revealed that Cdc42Hs, depleted of bound guanine nucleotide, was capable of tightly associating with the PC/PIP_2 vesicles, whereas Cdc42Hs containing bound GDP was capable of a weaker association with these vesicles. The GTPγS-bound Cdc42Hs showed no ability to associate with the PC/PIP_2 vesicles (*i.e.* relative to the background association observed with control PC vesicles). Thus, PIP_2 shows the same pattern of association with different guanine nucleotide-bound forms of Cdc42Hs as originally observed for the Dbl oncoprotein (Hart *et al.*, 1994).

It had been earlier reported that PIP_2 was able to stimulate the dissociation of [^{3}H]GDP from the Arf GTP-binding proteins (Terui *et al.*, 1994). Given our findings with Cdc42Hs, we were interested in determining whether PIP_2 might serve as a potential activator of other GTP-binding proteins, and in particular, other members of the Rho subfamily. As shown in Fig. 4, we found that PIP_2 was most effective on Cdc42Hs and RhoA, stimulating the dissociation of greater than 80% of the total bound [^{3}H]GDP within 5 min at room temperature. PIP_2 also was able to stimulate GDP dissociation from Rac1 (~50% of the total bound [^{3}H]GDP was dissociated after 5 min) but showed no ability to stimulate GDP dissociation from the Ha-Ras, Rap1a, or Ran GTP-binding proteins (Fig. 4), even when using PIP_2 levels as high as 0.5 mM. We also have found that PIP_2 will not stimulate [^{3}H]GDP dissociation from the K-Ras protein, under conditions where the Ras-GRF (Shou *et al.*, 1992) has a stimulatory effect (data not shown).

The ability of PIP_2 to regulate nucleotide binding to Rho subfamily proteins, as well as Arf, and to directly associate with Cdc42Hs (see Fig. 3) suggested that the Rho subfamily GTP-binding proteins must contain a specific PIP_2-binding site. Sequence comparisons between Rho subfamily GTP-binding proteins and two PIP_2-binding proteins, Gelsolin and Villin, indicated that the carboxyl-terminal domains of Cdc42Hs, RhoA, and the Rac proteins, which contain a number of basic amino acids, shared homology with amino acid residues 140–147 of Villin and 150–169 of Gelsolin. These regions of Villin and Gelsolin have been implicated in the binding of these proteins to PIP_2 (Janmey *et al.*, 1992). Thus, we constructed a deletion mutant of Cdc42Hs that lacked the carboxyl-terminal seven amino acids (including two arginines that were suspected to be involved in PIP_2 binding). This truncated Cdc42Hs molecule behaves like wild-type Cdc42Hs with regard to its intrinsic GTP-binding and GTPase activities and its ability to functionally couple to the Cdc42Hs-GTPase-activating protein (data not shown). Although it undergoes a slower rate of [^{3}H]GDP dissociation, compared to wild-type Cdc42Hs, it is still capable of interacting with Dbl and undergoing Dbl-catalyzed GDP dissociation (Fig. 5). However, the carboxyl-terminal truncated Cdc42Hs shows a markedly reduced response to PIP_2. These results then strongly argue that although Dbl and PIP_2 elicit similar effects (*i.e.* stimulation of GDP dissociation), they mediate these common effects from distinct binding domains on the GTP-binding protein.

In addition to serving as a precursor for the second messengers IP_3 and diacylglycerol (Berridge, 1993) and for the putative messenger PIP_3 (Stephens *et al.*, 1993), PIP_2 has been implicated as a regulator of the actin cytoskeleton, based on its ability to influence actin severing, capping, and bundling proteins *in vitro* (Janmey, 1994). Thus, it is interesting that in the present studies, we find that PIP_2 binds directly to and influences the nucleotide state of GTP-binding proteins that have been implicated in cytoskeletal regulation, *i.e.* Cdc42Hs and RhoA. However, these findings raise a number of important issues. One has to do with the mechanism by which PIP_2 stimulates GDP dissociation and how this compares with the mechanism by which Dbl stimulates GDP dissociation and guanine nucleotide exchange. Certainly the rate-limiting step in the activation of GTP-binding proteins, which occurs as an

outcome of the exchange of GTP for bound GDP, is the dissociation of the tightly bound GDP molecule. Both Dbl and PIP_2 strongly catalyze this dissociation event and stabilize the nucleotide-depleted state of the GTP-binding protein. Our data, in fact, would suggest that PIP_2 does this even more effectively than Dbl, such that PIP_2 can stimulate GDP dissociation from the nucleotide binding site of Cdc42Hs in the absence of added GTP, whereas Dbl cannot. It is possible that the differences exhibited by PIP_2 and Dbl reflect differences in the sites on Cdc42Hs (or related proteins) that bind these agents. At the present time, we know very little about the specific sites on Cdc42Hs that are responsible for binding Dbl, although mutations in Cdc42Hs that correspond to mutations in Ras that uncouple its binding to the GEF Sos (Mosteller *et al.,* 1994) do not uncouple Cdc42Hs from Dbl.[3] We would speculate at this point that a key conformational change that is necessary to loosen the binding of GDP to Cdc42Hs is induced by both Dbl and PIP_2 from distinct (binding) sites on the Cdc42Hs molecule. Future studies will be aimed at obtaining additional information regarding the conformational change in Cdc42Hs and related Rho subfamily proteins that is necessary for this rate-limiting step for activation.

A second key issue raised by these studies concerns whether, in fact, PIP_2 acts as a physiological regulator of Cdc42Hs and related proteins, and if so, how? It is tempting to speculate that the actin-regulatory activities of PIP_2 are related to the actions of Cdc42Hs and RhoA in mediating cytoskeletal changes such as filopodia formation and/or actin stress fiber formation. This is a particularly interesting possibility given the suggestions that a cascade of Rho subfamily GTPases (*i.e.* Cdc42Hs, Rac1, and RhoA) is operating in the regulation of cytoskeletal changes in certain cells (Nobes and Hall, 1995) and that a putative target for Cdc42Hs, the phosphatidylinositol 3-kinase (Zheng *et al.,* 1994a), which generates phosphatidylinositol compounds phosphorylated at the 3 position, may be upstream from Rac1 (Hawkins *et al.,* 1995; Nobes *et al.,* 1995). Thus, in addition to Dbl-related proteins, it is possible that phosphatidylinositol metabolites might serve as direct regulators of a signaling cascade that lead to changes in the actin cytoskeleton. It also is possible that phosphatidylinositol compounds work together (cooperatively) with Dbl-related proteins to activate GTP-binding proteins. We have not detected any type of cooperativity between oncogenic Dbl and PIP_2 in the stimulation of GDP dissociation from Cdc42Hs (data not shown). However, in some cases, Dbl-related proteins have been shown to bind to Rac without stimulating GDP dissociation (Miki *et al.,* 1993; Horii *et al.,* 1994), and in other cases, the presence of Dbl homology domains within proteins (*e.g.* the Ras-GEFs, Sos and Ras-GRF) have not yet been assigned a function (Shou *et al.,* 1992). Thus, it will be interesting to see if phosphatidylinositol compounds exert some type of regulatory or cooperative effect on the functions of these Dbl-related proteins. Finally, studies with purified phospholipase D indicate that the maximum activation of at least one isoform of the enzyme requires PIP_2, and the synergistic actions of the Arf GTP-binding protein (which also binds and is regulated by PIP_2 (Terui *et al.,* 1995)) and either RhoA or Cdc42Hs (Singer *et al.,* 1995). In the future, we intend to examine these possibilities further as well as determine just how PIP_2 levels in cells might be coordinated with the activation-deactivation cycle of Cdc42Hs and/or RhoA. For example, it is possible that signaling pathways that lead to an increase in PIP_2 levels will also promote the generation of guanine nucleotide-depleted Cdc42Hs and/or RhoA. Situations which then lead to a decrease in PIP_2 levels (*e.g.* stimulation of the hydrolysis of PIP_2 by phospholipase C enzymes) would then enable cellular GTP to bind to these GTP-binding proteins, thereby stimulating their activation. However, it also is possible that at the cellular levels of GTP (>10 μM), the exchange of GDP for GTP can occur even in the presence of high concentrations of PIP_2. Thus far, it has been difficult to test these levels of GTP in conventional GTP-binding assays; however, we hope in the future to be able to use fluorescence spectroscopic approaches to determine if, in fact, such a PIP_2-stimulated nucleotide exchange reaction is feasible.

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[3] Y. Zheng, unpublished results.

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Lfc and Lsc Oncoproteins Represent Two New Guanine Nucleotide Exchange Factors for the Rho GTP-binding Protein*

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Lfc and Lsc are two recently identified oncoproteins that contain a Dbl homology domain in tandem with a pleckstrin homology domain and thus share sequence similarity with a number of other growth regulatory proteins including Dbl, Tiam-1, and Lbc. We show here that Lfc and Lsc, like their closest relative Lbc, are highly specific guanine nucleotide exchange factors (GEFs) for Rho, causing a >10-fold stimulation of [^{3}H]GDP dissociation from Rho and a marked stimulation of GDP-[^{35}S]GTPγs (guanosine 5′-*O*-(3-thiotriphosphate) exchange. All three proteins (Lbc, Lfc, and Lsc) are able to act catalytically in stimulating the guanine nucleotide exchange activity, such that a single molecule of each of these oncoproteins can activate a number of molecules of Rho. Neither Lfc nor Lsc shows any ability to stimulate GDP dissociation from other related GTP-binding proteins such as Rac, Cdc42, or Ras. Thus Lbc, Lfc, and Lsc appear to represent a subgroup of Dbl-related proteins that function as highly specific GEFs toward Rho and can be distinguished from Dbl, Ost, and Dbs which are less specific and show GEF activity toward both Rho and Cdc42. Consistent with these results, Lbc, Lfc, and Lsc each form tight complexes with the guanine nucleotide-depleted form of Rho and bind weakly to the GDP- and GTPγS-bound states. None of these oncoproteins are able to form complexes with Cdc42 or Ras. However, Lfc (but not Lbc nor Lsc) can bind to Rac, and this binding occurs equally well when Rac is nucleotide-depleted or is in the GDP- or GTPγS-bound state. These findings raise the possibility that in addition to acting directly as a GEF for Rho, Lfc may play other roles that influence the signaling activities of Rac and/or coordinate the activities of the Rac and Rho proteins.

The Dbl family constitutes a group of oncoproteins and growth regulatory factors that have been implicated in a diversity of biological responses. Among the members of this family is the prototype Dbl oncoprotein, as well as Cdc24, a *Saccharomyces cerevisiae* cell-division-cycle protein involved in bud-site assembly (1, 2), Bcr, the breakpoint cluster region protein that has been implicated in the development of certain human leukemias (3), Tiam-1, which was first identified as a gene product involved in cell invasiveness and metastasis (4), and the *vav* (5), *ost* (6), *ect2* (7), *tim* (8), *fgd1* (9), *abr* (10), *dbs* (11), *lbc* (12), *lfc* (13), and *lsc* (14) oncogene products. The Dbl oncoprotein was first discovered when transfecting the DNA from diffuse B cell lymphomas into NIH 3T3 fibroblasts (1). Analysis of the primary amino acid sequence of the Dbl protein indicated that it contained a region of ~250 amino acids that shared homology with Cdc24 and Bcr. Given that genetic evidence placed Cdc24 upstream of Cdc42 in the bud-site assembly pathway in *S. cerevisiae*, it seemed plausible that Dbl regulated the actions of the human Cdc42 protein (Cdc42Hs). This led to the biochemical demonstration that Dbl is a guanine nucleotide exchange factor (GEF)[1] for Cdc42Hs and Rho (15, 16) and that Cdc24 is a highly specific GEF for the *S. cerevisiae* Cdc42 protein (Cdc42Sc) (2). It also was shown that the region of sequence similarity that Dbl shared with Cdc24 was critical for both GEF activity and for cellular transformation (16). More recent sequence analysis has subdivided this region of sequence similarity into two domains that are shared among all of the members of the Dbl family. The first domain, designated the Dbl homology (DH) domain, is essential for the GEF activity of Dbl, and the second domain, which shares homology with the platelet protein pleckstrin (designated the PH domain), is critical for the proper cellular targeting of Dbl and related proteins (13, 17).

Based on the initial biochemical studies performed on Dbl and Cdc24, it has been generally assumed that all proteins that contain a DH domain-PH domain in tandem will be GEFs for Rho-subtype proteins. In some cases this has been borne out. For example, Tiam-1 shows *in vitro* GEF activity toward Rac, Rho, and Cdc42 (18), and Lbc is a specific GEF for Rho (19). However, in a number of other cases, no GEF activity has yet been associated with the Dbl-related protein. This raises some important questions. 1) Are all DH domains involved in GEF activity or do they serve other biological roles, for example in the recruitment of Rho-related GTP-binding proteins to particular cellular locations and/or signaling complexes? 2) What accounts for the presumed specificity in the functional coupling of Rho-like proteins to Dbl-related molecules? In some cases *in vivo* specificity is probably mediated by cellular targeting, as accomplished by the individual PH domains of the different Dbl proteins. However, there also are clear indications that certain DH domains couple with high specificity to GTP-binding proteins, as exemplified by the interaction between Lbc and Rho. Overall, a better understanding of the regulation of Rho-re-

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[1] The abbreviations used are: GEF, guanine nucleotide exchange factor; DH, Dbl homology; GST, glutathione *S*-transferase; JNK, c-Jun kinase; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Mant, *N*-methylanthraniloyl; PH, pleckstrin homology.

Appendix 4

lated GTP-binding proteins by members of the Dbl family is of broad significance, given the highly coordinated actions of the Rho proteins in mediating a series of cytoskeletal alterations including filopodia and lamellipodia formation and the generation of stress fibers (20–23), as well as in stimulating DNA synthesis (24–29) and promoting cell morphology changes and cell motility (30).

In the present study, we have begun to address the questions raised above concerning the functions of Dbl-related proteins. To do this we have taken advantage of what appears to be a subgroup within the Dbl family, for which the prototype is Lbc and which includes the recently identified Lfc and Lsc oncoproteins. Lfc and Lsc (*i.e.* the "first" and "second" cousins of Lbc) were initially identified along with Dbs ("Dbl's big sister"), as the products of cDNAs that induce transformation, by using a retroviral vector-based expression system to transfer a library of cDNAs from the murine 32D or B6SUtA$_1$ hematopoietic cell lines into NIH 3T3 fibroblasts (31). Both Lfc and Lsc share the highest sequence similarity with the Lbc oncoprotein, within the regions of the DH and PH domains. In this work, we first set out to determine if like Lbc, the Lfc and Lsc proteins were capable of stimulating guanine nucleotide exchange on Rho-related proteins. We also were interested in determining if Lfc and Lsc demonstrated a high degree of specificity in their binding and GEF activities. The demonstration that Lfc and Lsc, as well as Lbc, were highly specific in their interactions with Rho proteins could lead to new insights regarding the elements within DH domains that impart the ability to recognize individual members of the Rho family.

EXPERIMENTAL PROCEDURES

Expression and Purification of Proteins—The GST-Lsc and GST-Lfc proteins were prepared by first inserting the cDNAs encoding a fragment of Lfc (fragments 208–573) and Lsc (fragments 333–778), which encompasses the DH and PH domains of these proteins, into the pGEX2T vector, and then the glutathione *S*-transferase (GST) constructs were cloned into the *Bam*HI site of pAX142 (13, 14). The GST constructs were digested from pAX142 at the *MluI*/*Sal*I sites; the fragments were then blunt-ended and inserted into the *MluI*/*Sma*I sites of the baculovirus transfer vector pVL1393. *Spodoptera frugiperda* cells (SF21) were infected with recombinant baculovirus; the cells were collected at 48 h postinfection and lysed in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The GST fusion proteins were purified by glutathione-agarose affinity chromatography. Preparation of GST-Cdc24 and GST-Lbc and their expression in SF21 cells has been previously described (2, 19) as has the expression of GST-GRF in *Escherichia coli* (32). The GTP-binding proteins RhoA, Rac, Cdc42, and Ras were expressed as His-fusion proteins in *E. coli*. The construction of an expression vector containing GST fused to the cDNAs encoding the full-length genes for different Ras-related GTP-binding proteins has been described (16, 19). For RhoA, Rac, Ras, and Cdc42, the cDNAs were transferred into the *Bam*HI/*Eco*RI sites of a modified pET15b vector that allowed the coding region to be in frame with the upstream hexa-His tag. The plasmid was transformed into BL21 (DE3) *E. coli*, and an overnight culture from a single colony was used to inoculate a 1-liter culture that was grown at 37 °C, while shaking, to an A_{560} of 0.6 (this took approximately 4 h). At this time, the protein expression was induced by the addition of 200 μM isopropyl-β-D-thiogalactoside for 2 h. Bacteria were harvested by centrifugation and frozen at −80 °C. The pellets were thawed in lysis buffer consisting of 20 mM Tris-HCl (pH 8.0), 5 mM imidazole, 500 mM NaCl, 1 mM sodium azide, 200 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, and resuspended using a glass/Teflon Dounce homogenizer. The bacteria were lysed on ice by adding 0.5 mg/ml lysozyme with 5 mM EDTA followed by 10 μg/ml DNase I (Boehringer Mannheim) and $MgCl_2$ to 10 mM. The lysate was cleared by centrifugation for 30 min at 30,000 rpm. The proteins were purified by affinity chromatography, using an iminodiacetic acid agarose column, charged with $NiSO_4$. The 17-amino acid hexa-His tag was then removed by thrombin cleavage. In all assays, the GST fusion proteins were used without the removal of GST. The amounts of the GEFs used in all experiments were estimated by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis.

GDP/GTP Exchange Assays—The GDP dissociation and GTP binding assays were carried out by the filter binding method at 24 °C as described previously (15). The quantities of GTP-binding proteins and the amounts of GST, GST-Lsc, GST-Lfc, GST-Lbc, GST-Cdc24 and GST-GRF used for each individual experiment are indicated in the figure legends. In the initial screens to detect guanine nucleotide exchange activity, the GTP-binding proteins were loaded with [^{3}H]GDP and incubated with control and test proteins. After 15 min, the samples were quenched with ice-cold dilution buffer, containing 10 mM $MgCl_2$, and collected by filter binding and counted to determine the relative amount of bound [^{3}H]GDP remaining. To further characterize potential nucleotide exchange activities detected in the initial screen, a full time course of [^{3}H]GDP dissociation and [^{35}S]GTPγS binding was performed.

Fluorescence Spectroscopy—Fluorescence measurements were made using an SLM 8000C spectrofluorimeter in the photon counting mode. Samples were stirred continuously and thermostated at 25 °C in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$. *N*-Methylanthraniloyl (Mant)-dGTP was synthesized according to the published procedure (33) from dGTP and *N*-methylisatoic acid (Molecular Probes, Eugene OR). Guanine nucleotide exchange assays were carried out by initially incubating 650 nM RhoA with 450 nM Mant-dGTP and monitoring Mant fluorescence (excitation = 350 nm, emission = 440 nm). Exchange of Mant-dGTP for GDP on RhoA was then initiated by the addition of either GST-Lbc, GST-Lfc, or GST-Lsc, so that the final GEF concentration varied between 25 and 100 nM. 200 s after nucleotide exchange was initiated, EDTA was added to a final concentration of 6.7 mM, thus allowing the exchange of Mant-dGTP for GDP on RhoA to be driven to completion; this was done to demonstrate that equal amounts of RhoA bound to Mant-dGTP were present in each sample. The initial rates for the nucleotide exchange activities catalyzed by Lbc, Lfc, or Lsc were estimated by applying linear fits to the first 50 s after the addition of the GEF, using IgorPro wavemetrics software.

Complex Formation of His-GTP-binding Proteins with GST-Lsc and GST-Lfc—Interactions between G-proteins and GEFs were detected *in vitro* by using immobilized GST-Lsc and GST-Lfc, and as positive controls, GST-Lbc, GST-Cdc24, and GST-GRF were bound to glutathione-agarose beads to co-precipitate purified His-tagged (clipped) GTP-binding proteins. Initially all interactions were assayed using the nucleotide-free state of the GTP-binding proteins. Co-precipitations were performed in 500-μl volumes of 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (incubating for 2 h at 4 °C). The agarose beads were washed in the precipitation buffer three times by centrifugation and resuspended in Laemmli buffer, subjected to SDS-polyacrylamide gel electrophoresis, and Western blotted. Co-precipitations were performed for each GTP-binding protein individually, and interactions were detected using anti-RhoA monoclonal antibody (Santa Cruz Biotechnology), or anti-Rac polyclonal antibody (Santa Cruz Biotechnology), anti-Ras monoclonal antibody (Santa Cruz Biotechnology), or anti-Cdc42 antibody (raised against the carboxyl-terminal 23 amino acids), by the ECL method (Amersham Corp.).

In order to fully characterize the nucleotide dependence of the interactions occurring between GEFs and GTP-binding proteins in the nucleotide-free state, we repeated co-precipitations using RhoA and Rac bound to GDP and GTPγS. These experiments were performed as described above except that the GTP-binding proteins were preloaded with the appropriate nucleotide, and the EDTA was replaced with 10 mM $MgCl_2$ in order to stabilize nucleotide binding to the GTP-binding proteins.

RESULTS

The principal aim of these studies was to determine whether the Lfc and Lsc proteins were capable of functionally coupling to the members of the Rho subfamily of GTP-binding proteins. Previous studies have shown that the DH domain of Dbl is essential for both its GEF activity and transforming capability (16). All members of the Dbl family also contain a PH domain, which is immediately carboxyl-terminal to the DH domain, and recent findings suggest that the PH domain is important for cellular targeting rather than for GEF activity (13, 17). However, because of the possibility that the PH domains of Dbl proteins may have other regulatory functions, and because we have found that the presence of surrounding sequences includ-

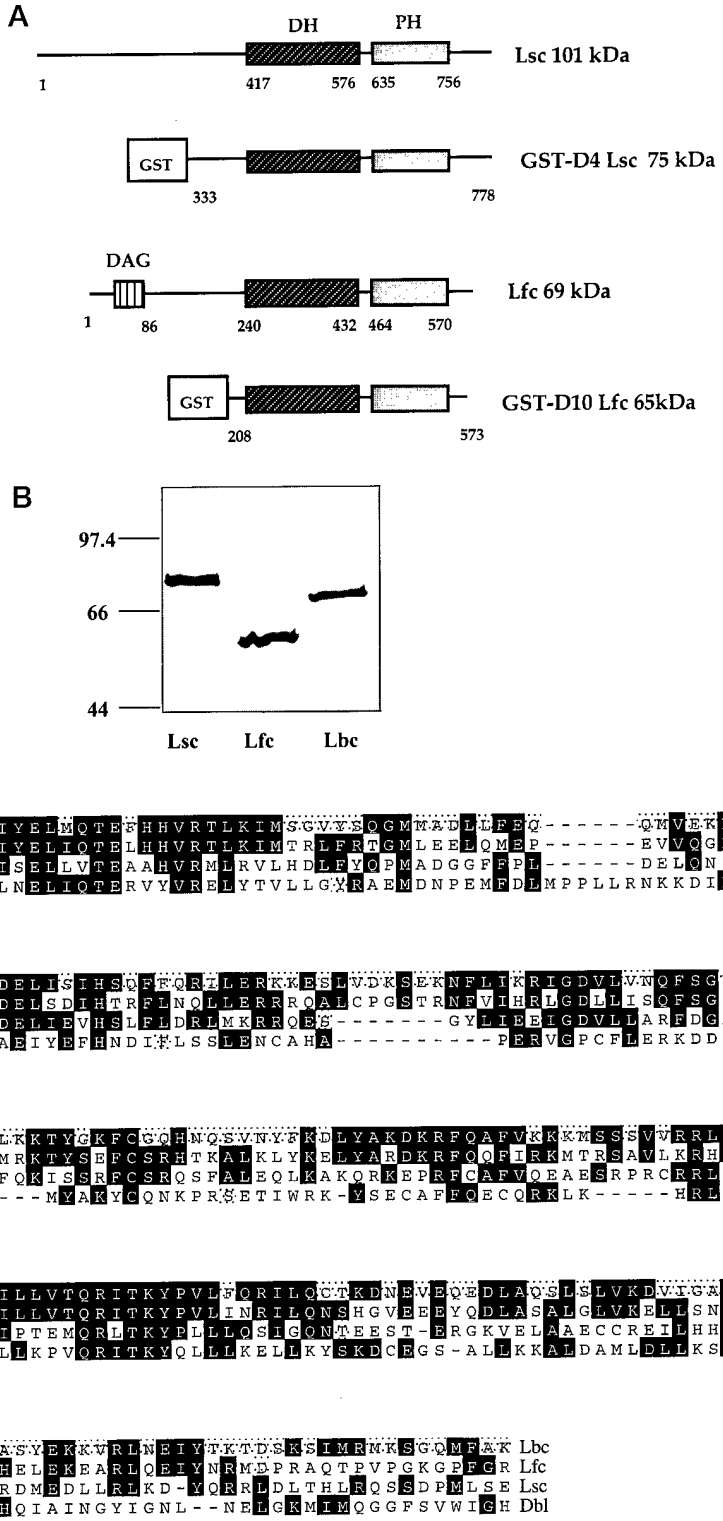

FIG. 1. **Expression of Lfc and Lsc as GST fusion proteins.** *A*, schematic representation of full-length Lfc and Lsc and the GST-Lfc and GST-Lsc fusion proteins that were expressed and assayed for GEF activity. D4 Lsc and D10 Lfc refer to constructs that have been described previously (13, 14). *B*, expression and purification of Lfc, Lsc, and Lbc as GST fusion proteins. SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) of the purified GST fusion proteins which were prepared from SF21 insect cell lysates infected with recombinant viruses that encode the Lfc, Lsc, and Lbc proteins. *C*, comparison of the Dbl homology domains from Lbc, Lfc, Lsc, and Dbl. Alignment of residues 78–304 of Lbc to similar sequences of Lfc, Lsc, and Dbl is shown. *Boxed* residues represent sequence identities.

ing the PH domain may be necessary to achieve the proper folding of DH domains, we expressed and purified forms of the Lfc and Lsc proteins that include both the DH and PH domains, as well as some additional flanking sequences. Fig. 1*A* shows the schematic representations of both the full-length Lfc and Lsc proteins and the glutathione *S*-transferase (GST) fusion proteins that were expressed in *S. frugiperda* (SF21) cells and assayed for GEF activities (see below). Fig. 1*B* shows the SDS-polyacrylamide gel electrophoretic profiles of the GST-Lfc and GST-Lsc proteins. Both of the proteins could be highly purified by glutathione-agarose chromatography and appeared to be fully soluble.

Determination of Guanine Nucleotide Exchange Activity for Lfc and Lsc—Comparisons of the Dbl domains of Lfc and Lsc with other members of the Dbl family show that these domains are most similar to those for Lbc (Fig. 1*C*). Given that Lbc is a highly specific GEF for Rho, we examined whether the Lfc and Lsc proteins were capable of similar biochemical activities. Fig. 2, *A* and *B*, shows that this is the case. Both Lfc (Fig. 2*A*) and Lsc (Fig. 2*B*) were highly effective in stimulating the dissociation of [^{3}H]GDP from *E. coli*-expressed RhoA. In the absence of any regulatory protein, the half-time for the dissociation of

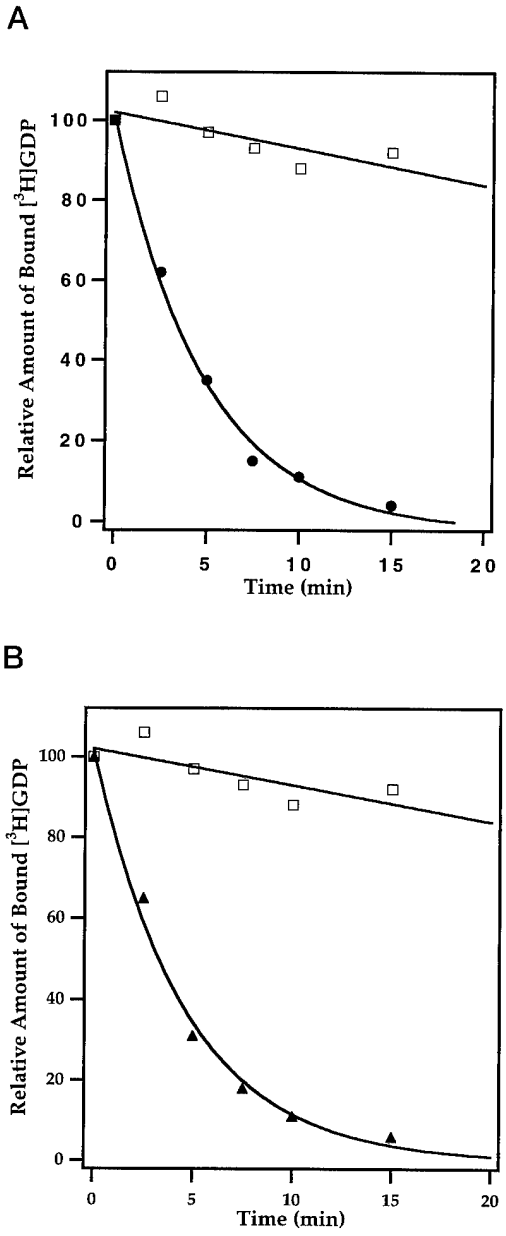

FIG. 2. **Stimulation of GDP dissociation from RhoA by Lfc and Lsc.** *A*, measurement of the dissociation of [^{3}H]GDP from the *E. coli* expressed RhoA protein as stimulated by GST-Lfc. *B*, measurement of the dissociation of [^{3}H]GDP from the *E. coli* expressed RhoA protein as stimulated by GST-Lsc. For each experiment, 2 μg of recombinant RhoA protein were preloaded with [^{3}H]GDP and then added to incubations containing 5 μg of GST (□), 1 μg of GST-Lfc (●), or 1 μg of Gst-Lsc (▲) in reaction buffer containing 100 μM cold GTP for the indicated time before terminating the reactions by the nitrocellulose filter binding method (see "Experimental Procedures").

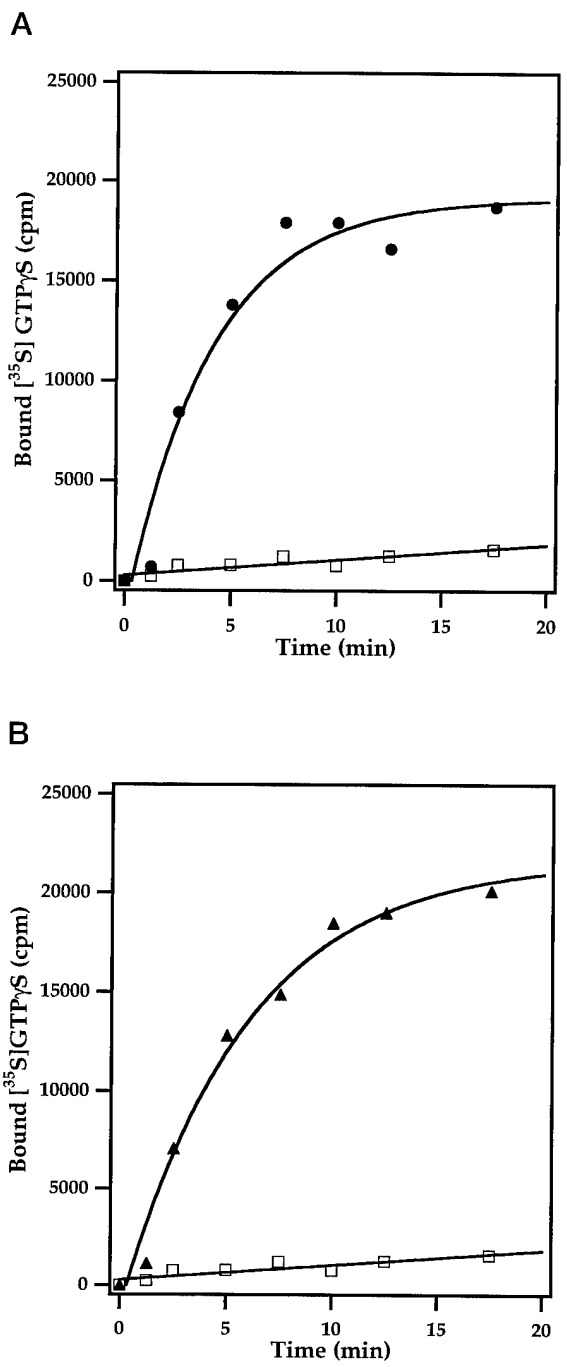

FIG. 3. **Stimulation of GTPγS binding to RhoA by Lfc and Lsc.** *A*, measurement of GST-Lfc-stimulated GTPγS binding to *E. coli* expressed RhoA protein. *B*, measurement of GST-Lsc-stimulated GTPγS binding to *E. coli* expressed RhoA protein. For each experiment, 5 μg of GST (□), 1 μg of Gst-Lfc (●), or 1 μg of GST-Lsc (▲) were added to 2 μg of GDP-bound RhoA in a reaction mixture containing [^{35}S]GTPγS for the indicated time before termination of the reactions by the nitrocellulose filter binding method.

GDP from RhoA is relatively slow, *i.e.* >30 min at room temperature. However, both Lfc and Lsc proteins were able to markedly accelerate the rate of GDP dissociation by at least 10-fold such that the half-time for GDP dissociation was 2–3 min in the presence of these regulatory proteins.

Fig. 3, *A* and *B*, shows that the Lfc and Lsc proteins also strongly stimulated the exchange of GDP for [^{35}S]GTPγS. Here again in the absence of any regulatory factor, RhoA is capable of little if any guanine nucleotide exchange over a period of 20 min. However, both Lfc and Lsc catalyzed the complete exchange of GDP for GTPγS within ~10 min, thus indicating that these proteins qualify as effective GEFs for the RhoA protein.

We have closely compared the initial rates of guanine nucleotide exchange on RhoA that are catalyzed by Lfc, Lsc, and Lbc, under conditions where RhoA was present in at least a 6-fold excess over the GEFs, using a very sensitive fluorescence spectroscopic assay (Fig. 4*A*). This assay is based on the finding that the fluorescence emission of Mant-GTP is enhanced upon binding to GTP-binding proteins. Thus, under conditions where guanine nucleotide exchange is catalyzed, the presence of Mant-dGTP in a cuvette solution containing RhoA will result in an exchange of the GDP that was originally bound to RhoA for the Mant-nucleotide and thereby provide a real-time assay for

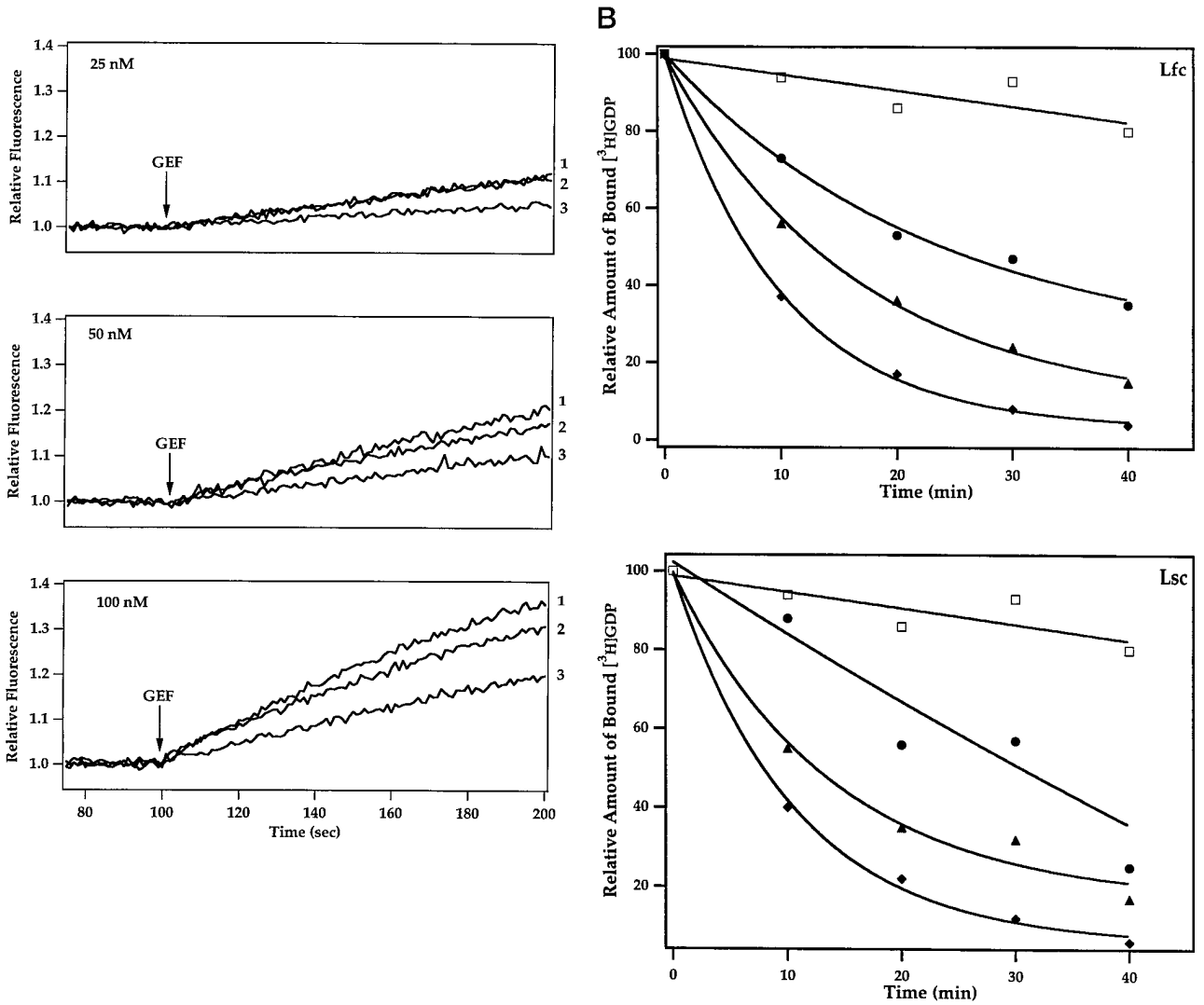

FIG. 4. **Comparison of Lfc, Lsc and Lbc: initial rates of exchange on RhoA and catalytic potential.** *A*, fluorescence spectroscopic analysis of initial rates for guanine nucleotide exchange on RhoA (650 nM) catalyzed by Lbc (*1*), Lfc (*2*), and Lsc (*3*) at 25, 50, and 100 nM. The rate of change of fluorescence was estimated using linear fits to the first 50 s after addition of GEFs. *B*, kinetics of guanine nucleotide exchange catalyzed by Lfc (*top panel*) and Lsc (*bottom panel*), using equimolar concentrations of RhoA and GEF (◆); ▲ represents a 2-fold excess of RhoA to GEF; ● represents a 4-fold excess of RhoA to GEF, and □ represents the GST control.

the exchange event, as monitored by the enhancement of Mant fluorescence. Fig. 4*A* shows that the purified recombinant Lfc, Lsc, and Lbc proteins each stimulated the enhancement of Mant fluorescence as an outcome of catalyzed GDP-Mant-dGTP exchange on RhoA. The time courses for the fluorescence changes stimulated by Lbc and Lfc were virtually identical for each of the three different GEF concentrations assayed. While we consistently found Lsc to stimulate guanine nucleotide exchange at a rate that was ~2-fold slower compared with the rate of exchange stimulated by Lbc and Lfc, it is difficult to know how much significance to attach to these differences because of the difficulties in estimating the protein concentrations for the amount of functional GEF present in the assay.

In order to further compare the catalytic potential of the Lfc and Lsc proteins, we assayed [^{3}H]GDP dissociation from RhoA as catalyzed by different concentrations of these GEFs. We found that guanine nucleotide exchange occurred rapidly when the GEF and GTP-binding proteins were present in equimolar concentrations. As expected, decreasing the concentration of Lfc or Lsc such that RhoA was in 2- or 4-fold excess (over [GEF]) resulted in slower half-times of dissociation. However, it appeared that complete dissociation of [^{3}H]GDP from RhoA will occur at each of the concentrations of Lfc and Lsc assayed, indicating that both of these proteins act catalytically in stimulating the guanine nucleotide exchange reaction.

Specificity of Lfc and Lsc as GEFs—The Lbc protein was shown to be a highly specific GEF for Rho and did not stimulate the guanine nucleotide exchange activity of Cdc42Hs, Rac, or Ras (19). Thus, we examined whether Lfc and Lsc showed similar GEF specificity. The data presented in Fig. 5 indicate that like Lbc, both Lfc and Lsc are highly specific for RhoA when assaying [^{3}H]GDP dissociation after 15 min at room temperature. Neither Lfc nor Lsc showed any GEF activity toward Ras under these conditions, whereas recombinant GST-Ras-GRF strongly stimulated GDP dissociation from Ha-Ras. Similarly neither Lfc nor Lsc showed any activity toward Cdc42Hs, again under conditions where Cdc24 effectively stimulated GDP dissociation. Both Lfc and Lsc were ineffective in stimulating GDP dissociation from Rac. The inability of Lfc and Lsc to serve as GEFs for Ras, Cdc42Hs, and Rac also was observed when complete time courses for [^{3}H]GDP dissociation were obtained (*i.e.* between 2.5 and 30 min (data not shown)). It is interesting that although the DH domains of oncogenic Dbl

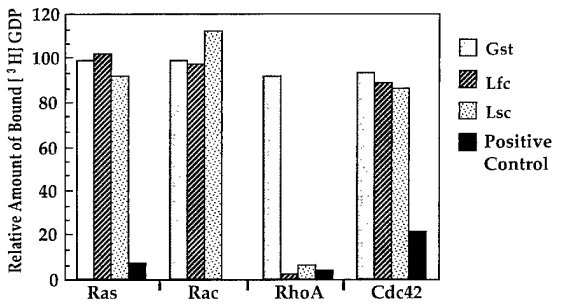

FIG. 5. **Specificity of Lfc and Lsc GEF activity on the Rho and Ras-type GTP-binding proteins.** 2 μg of the various recombinant GTP-binding proteins were preloaded with [^{3}H]GDP and incubated with 2 μg of GST, 1 μg of GST-Lfc, or 1 μg of GST-Lsc before termination of the reaction after 15 min. 1 μg of GST-Ras-GRF was used as a control for assaying stimulated dissociation of GDP from Ras; 1 μg of GST-Lbc was used as a control for stimulated GDP dissociation from RhoA, and 1 μg of Cdc24 was used as a control for stimulated GDP dissociation from Cdc42 and Rac.

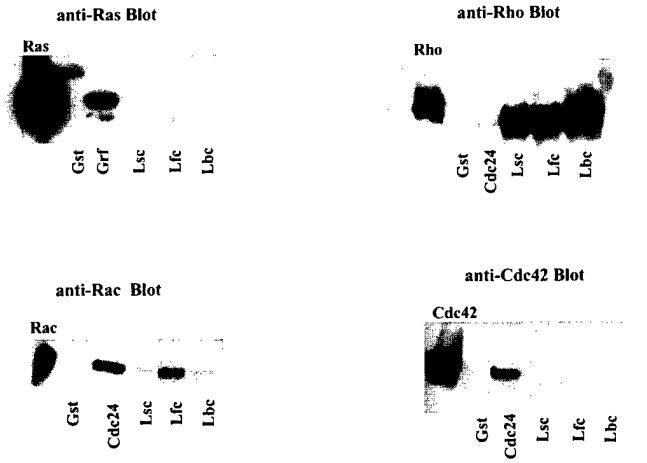

FIG. 6. **Interactions of Lfc and Lsc with the Rho and Ras-type GTP-binding proteins in the nucleotide-free state.** Specific association of Lfc and Lsc with various GTP-binding proteins was determined by using GST-Lfc and GST-Lsc bound to glutathione-agarose beads to precipitate the GTP-binding proteins. The GTP-binding proteins were depleted of nucleotide by preincubation with 10 mM EDTA, as described under "Experimental Procedures." Proteins bound to the beads were resolved by SDS-polyacrylamide gel electrophoresis (12%) and immunoblotted using antibodies directed against the GTP-binding proteins.

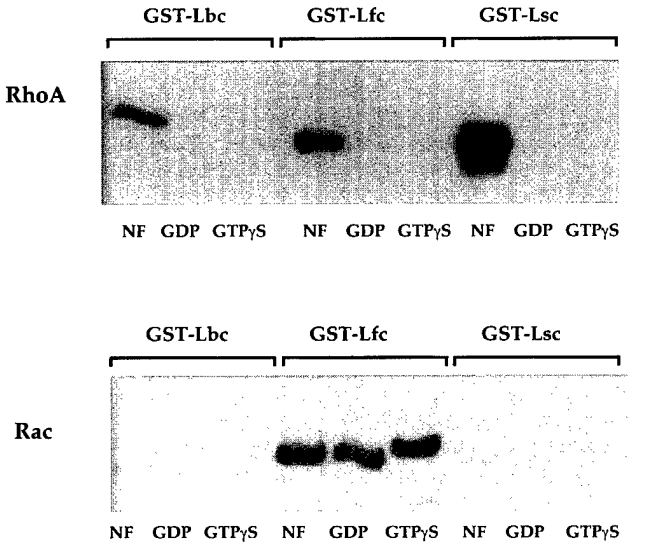

FIG. 7. **Specificity of the nucleotide state of RhoA and Rac for interactions with Lsc and Lfc.** Binding of Lfc, Lsc, and Lbc to RhoA and Rac was determined under different conditions of guanine nucleotide occupancy. Co-precipitation of recombinant RhoA or Rac with GST-Lfc, Lsc, and Lbc fusion proteins was carried out as described in Fig. 6. The nucleotide state of the GTP-binding proteins was established by preincubation in buffer containing 10 mM EDTA (to achieve the nucleotide-depleted state) or buffer containing 10 mM $MgCl_2$ and 200 μM GDP or 200 μM GTPγS for 30 min to establish the GDP-bound and GTPγS-bound states.

(16), Ost (6), and Dbs[2] are able to functionally couple to both Cdc42Hs and RhoA, apparently the related DH domains of Lbc, Lfc, and Lsc are only able to act as GEFs toward Rho proteins. This in turn suggests that the basic framework for GEF specificity toward Rho is contained within the DH domains of the Lbc subfamily of Dbl proteins.

Do Lfc and Lsc Show Similar Binding Specificity for Rho Family Members?—Some members of the Dbl family are able to bind to Rho-related GTP-binding proteins but do not stimulate their guanine nucleotide exchange activity (6, 7). This suggests that in some cases the DH domains serve as binding motifs, perhaps functioning only to recruit GTP-binding proteins. Following this line we wanted to examine whether Lbc, Lfc, and Lsc acted strictly as GEFs, such that they showed similar binding specificity as that exhibited in the GEF assays, or if one or more of these Dbl proteins within the Lbc subfamily were capable of binding to other GTP-binding proteins, in addition to Rho. These experiments were carried out using Lbc, Lfc, and Lsc expressed as GST fusion proteins and immobilized on glutathione-agarose beads. We first assayed the binding specificities of these proteins for different GTP-binding proteins in their guanine nucleotide-depleted state, since this should be the preferred state for binding to GEFs. Fig. 6 shows that as expected, GST-Lbc and GST-Lsc selectively associated with RhoA but not with Rac, Cdc42, or Ras. Also as expected, GST-Ras-GRF formed a complex with Ras and GST-Cdc24 complexed with Cdc42Hs. However, it was surprising that although GST-Lfc bound RhoA as effectively as Lbc and Lsc, the GST-Lfc protein also associated with Rac (as did GST-Cdc24).

We next examined the nucleotide specificity for the binding of RhoA and Rac to the Lbc subfamily members. The results presented in Fig. 7 show that Lbc, Lfc, and Lsc bound specifically to the guanine nucleotide-depleted state of RhoA, again, as is typically the case for GEFs (16). However, the interaction between Lfc and Rac did not demonstrate this specificity, such that Lfc effectively associated with both the GDP- and GTPγS-bound states of Rac as well as with the nucleotide-depleted form of the protein. Neither Lbc nor Lsc showed any binding capability for Rac, regardless of nucleotide state.

Based on the finding that Lfc was able to associate with both the GDP- and GTP-bound forms of Rac, we assayed the ability of Lfc to inhibit GDP dissociation (*i.e.* act as a GDP dissociation inhibitor) or influence GTP hydrolysis (data not shown). All such assays were negative, that is we found no detectable GDP dissociation inhibitor activity for Lfc nor did we find that Lfc could alter the intrinsic or GTPase-activating protein-stimulated GTP hydrolysis of the Rac protein.

DISCUSSION

The Lfc and Lsc oncoproteins are two recently discovered members of the Dbl family of growth regulatory proteins. Each of the members of this family share two motifs, a Dbl homology (DH) domain of ~150 amino acids and a pleckstrin homology (PH) domain that contains ~100 amino acids. Various members of this family including the prototype, Dbl, as well as Cdc24, Tiam-1, and Lbc have been shown to act as guanine

[2] J. A. Glaven, I. P. Whitehead, R. Kay, and R. A. Cerione, unpublished data.

nucleotide exchange factors (GEFs) by stimulating the exchange of GTP for GDP on Rho subfamily GTP-binding proteins (2, 15, 18, 19). In two cases, Cdc24 and Lbc, the GEF activity is highly specific, with Cdc42 serving as the substrate for Cdc24 and Rho serving as the substrate for Lbc. Given the fact that the DH domains of Lfc and Lsc are most similar to that of Lbc, we were interested in the possibility that these two oncoproteins might also act as specific GEFs for Rho and thus together with Lbc comprise a specific subgroup of the larger family of Dbl-related proteins. The data presented here suggest that this in fact is the case. Both Lfc and Lsc appear to be highly specific GEFs for Rho and show no detectable GEF activity toward Cdc42, Rac, or Ras. The abilities of these oncoproteins to stimulate GDP dissociation from or GDP-[^{35}S]GTPγS exchange on Rho are similar to the activities measured for Lbc, both with regard to the initial rate of GDP dissociation and the catalytic capability of their GEF activities. These findings then suggest that the essential features for GEF specificity for Rho are contained within the DH domains of Lbc, Lfc, and Lsc but are missing in the Dbl and Ost oncoproteins, since the latter two proteins functionally couple to Cdc42 as well as to Rho.

An obviously important question that is raised by these studies concerns the reason for the existence of multiple GEFs for Rho. One possibility might have been differences in tissue distribution; for example, if Lbc, Lfc, and Lsc showed markedly different tissue locations, then the need for multiple Rho GEFs would be obvious because of the ubiquitous distribution of Rho. However, the fact that all three of these oncoproteins appear to be located in similar tissues, and in the case of Lfc and Lsc, the same cell types, seems to argue against this explanation.

A second possibility may involve distinct cellular locations. There are already indications that one Rho subfamily member, Cdc42, is located both in the plasma membrane and in Golgi membranes (34) and that it may be necessary to activate Cdc42 at both of these cellular locations. The targeting of Dbl and Dbl-related proteins to specific cellular sites through their PH domains may provide a means to selectively activate Rho subfamily proteins at distinct locations. In the case of Dbl, the PH domain appears to target this GEF to a cytoskeletal location (17), whereas in the case of Lfc, the PH domain appears to be targeting the GEF to the plasma membrane, since replacement of the PH domain with a Ras-farnesylation sequence restores transforming activity to Lfc (13). The cellular locations of Lbc and Lsc have not yet been determined, although chances are that their PH domains will bind specific cellular targets. Thus, it will be interesting in the future to determine whether Lsc and Lbc are located in different regions of the cell relative to Lfc.

A third rather intriguing possibility for the existence of what appears to be multiple Rho GEFs concerns the potential involvement of these proteins in different signaling pathways mediated by other GTP-binding proteins (*i.e.* aside from Rho). This directly bears on the question of whether all proteins that contain DH domains act directly as GEFs or, if in some cases, these proteins serve to recruit GTP-binding proteins to a specific cellular site (as marked by the PH domain) where they can either be activated or regulated by other factors (*e.g.* lipids). There already is precedent for Dbl-related proteins binding GTP-binding proteins without having direct effects on guanine nucleotide exchange. One such example is Ect2 (7), which binds Rac in a guanine nucleotide-independent manner, and a second example is Ost (6), which associates specifically with GTP-bound Rac. In these studies, we show that Lfc, unlike Lbc or Lsc, binds to Rac in a guanine nucleotide-independent manner. This interaction does not appear to have any direct influence (either stimulatory or inhibitory) on GDP dissociation from Rac nor on GTP hydrolysis. However, it is interesting that recent work has shown that expression of Lfc (but not Lsc) in COS cells results in an activation of the c-Jun kinase (JNK1),[3] a nuclear mitogen-activated protein kinase that catalyzes the amino-terminal phosphorylation of c-Jun. Given that it has been well documented that Rac and Cdc42, but not Rho, initiate signaling cascades that culminate in the activation of JNK1 (24–27), it will be interesting to see if the Lfc-Rac interaction reported here is in some way involved in the pathway that mediates Lfc effects on JNK1 activity.

An important direction of future studies will be to further investigate the functional outcome of Lfc-Rac interactions. Recently, we have found that phosphatidylinositol 4,5-bisphosphate may represent an alternative factor for initiating the activation of Rho subtype GTP-binding proteins (35). Specifically, it appears that phosphatidylinositol 4,5-bisphosphate can bind directly to Cdc42 (and to lesser extents to Rac and Rho) and strongly stimulate GDP dissociation. While it does not appear that phosphatidylinositol 4,5-bisphosphate will act cooperatively with Dbl (since Dbl, alone, is fully capable of stimulating GDP-GTP exchange on Cdc42 or Rho), it will be interesting to see if phosphatidylinositol 4,5-bisphosphate or other lipids can act synergistically with Lfc to influence the activation state of Rac. The location of a potential lipid binding domain on Lfc raises other possibilities regarding lipid factors that might influence the functional coupling of this oncoprotein to Rho-like GTP-binding proteins. Thus, it may be that the family of Dbl-related proteins provide a broader group of functions than originally anticipated, not only acting directly as specific GEFs but also specifying the cellular sites where other factors can promote the activation of GTP-binding proteins and/or where targets can bind to GTP-binding proteins and mediate downstream signaling events.

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